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U.S. PATENT: ***9,708,361***
ISSUE DATE: ***July 18, 2017***

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Under Secretary of Commerce for Intellectual Property
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Certifying Officer

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(12) **United States Patent**
Watanabe et al.

(10) **Patent No.: US 9,708,361 B2**
(45) **Date of Patent: Jul. 18, 2017**

(54) **ANTISENSE NUCLEIC ACIDS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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US 2015/0166995 A1 Jun. 18, 2015

Related U.S. Application Data

(63) Continuation of application No. 13/819,520, filed as application No. PCT/JP2011/070318 on Aug. 31, 2011, now Pat. No. 9,079,934.

(30) **Foreign Application Priority Data**

Sep. 1, 2010 (JP) 2010-196032

(51) **Int. Cl.**

C07H 21/02	(2006.01)
C07H 21/04	(2006.01)
A61K 31/70	(2006.01)
C12N 15/11	(2006.01)
C12N 15/113	(2010.01)
C07H 21/00	(2006.01)
C12N 5/00	(2006.01)

(52) **U.S. Cl.**

CPC ***C07H 21/04*** (2013.01); ***C07H 21/00*** (2013.01); ***C12N 15/111*** (2013.01); ***C12N 15/113*** (2013.01); *C12N 2310/11* (2013.01); *C12N 2310/315* (2013.01); *C12N 2310/3145* (2013.01); *C12N 2310/321* (2013.01); *C12N 2310/3525* (2013.01); *C12N 2320/33* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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(57) **ABSTRACT**

The present invention provides an oligomer which efficiently enables to cause skipping of the 53rd exon in the human dystrophin gene. Also provided is a pharmaceutical composition which causes skipping of the 53rd exon in the human dystrophin gene with a high efficiency.

7 Claims, 19 Drawing Sheets

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Figure 1

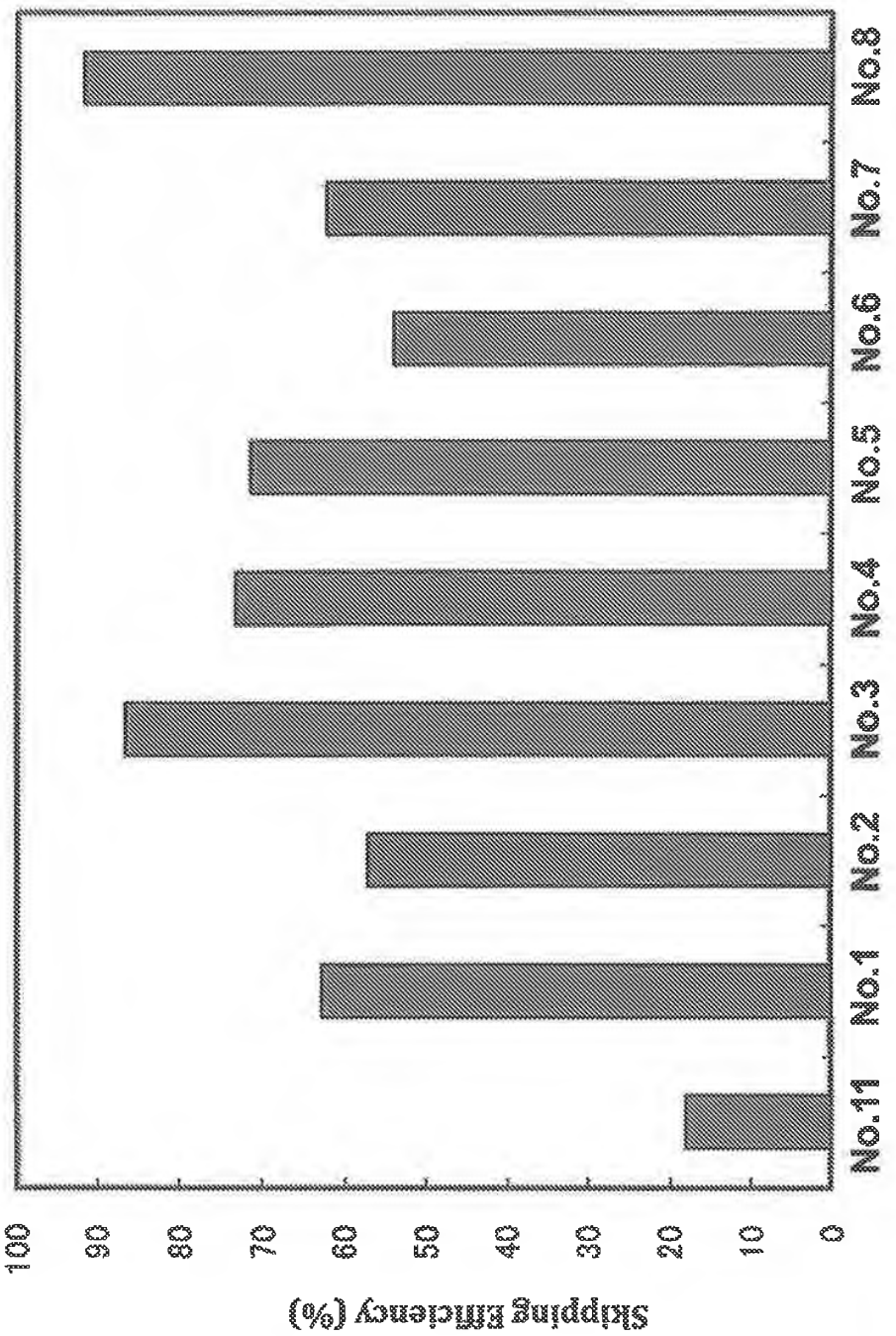

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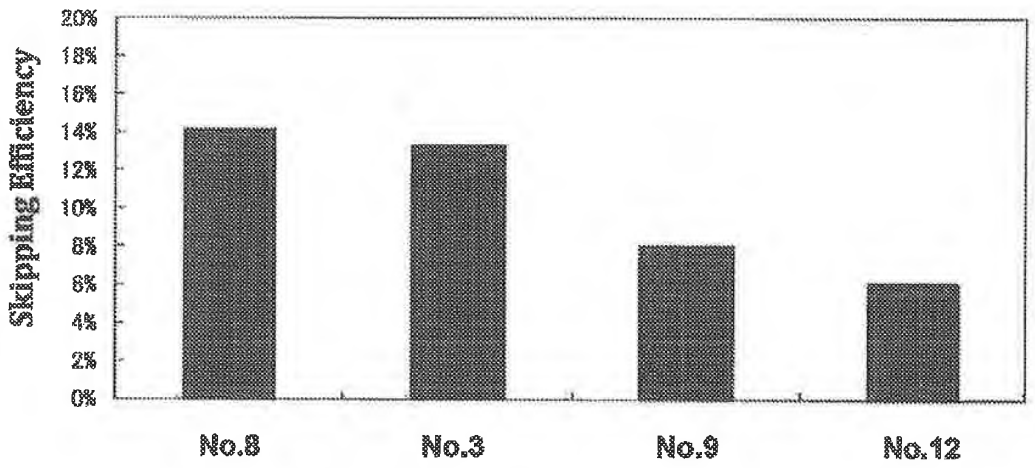
Figure 2



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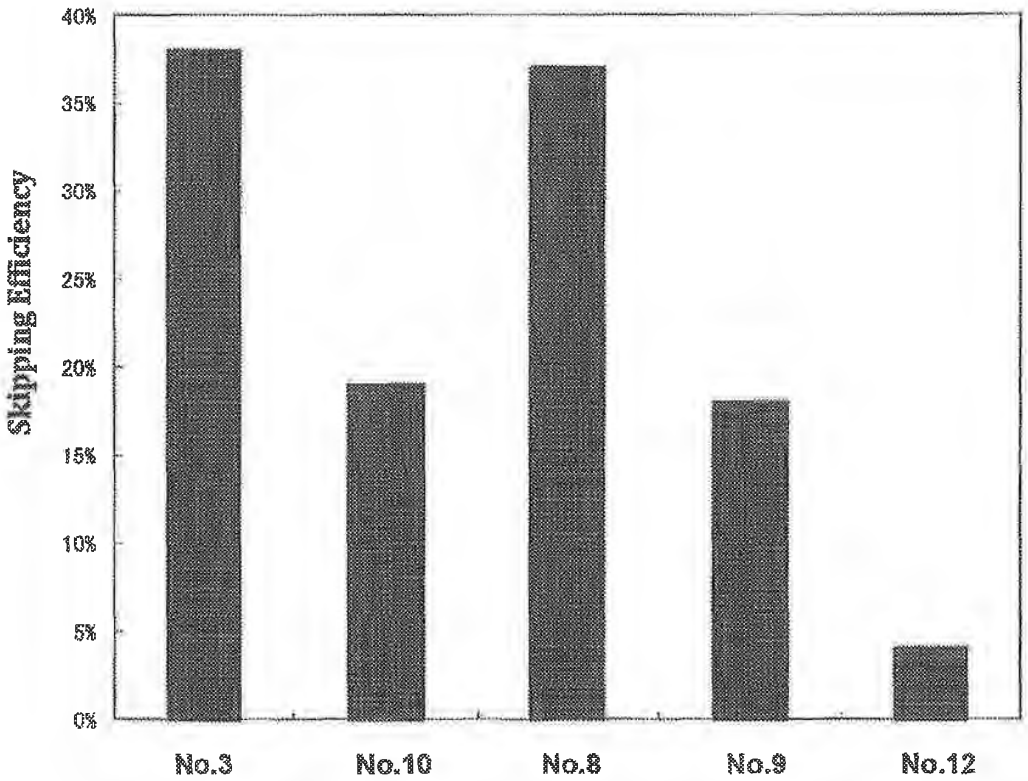

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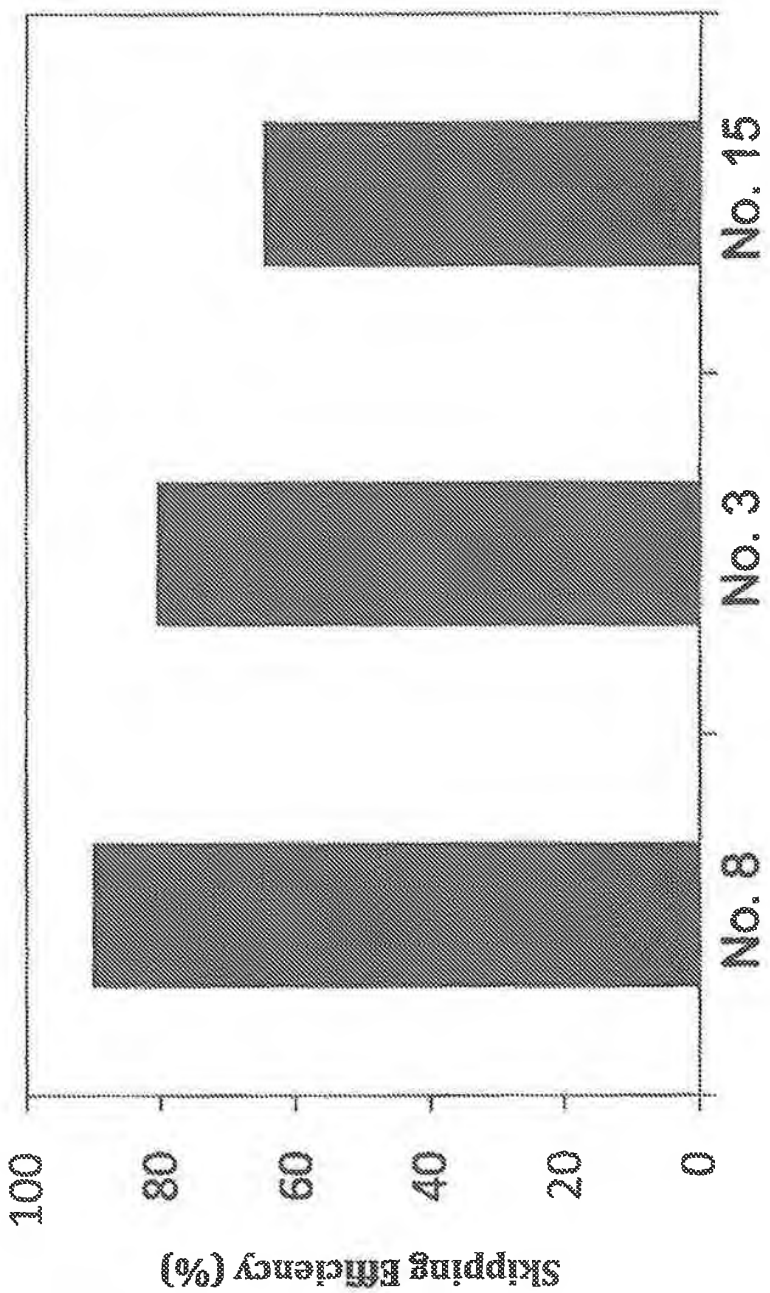

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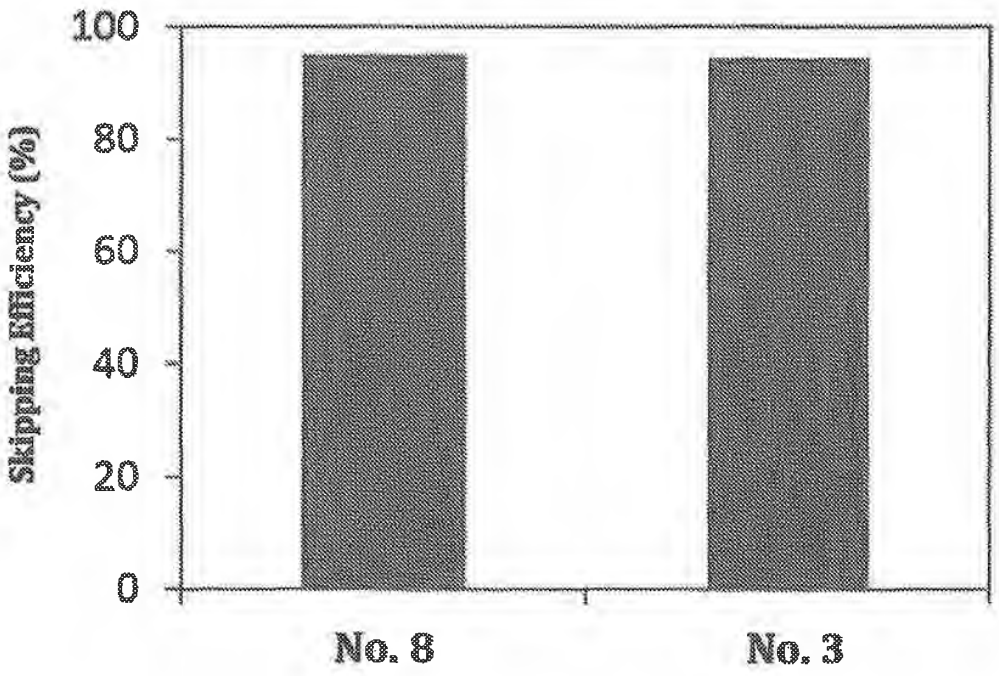

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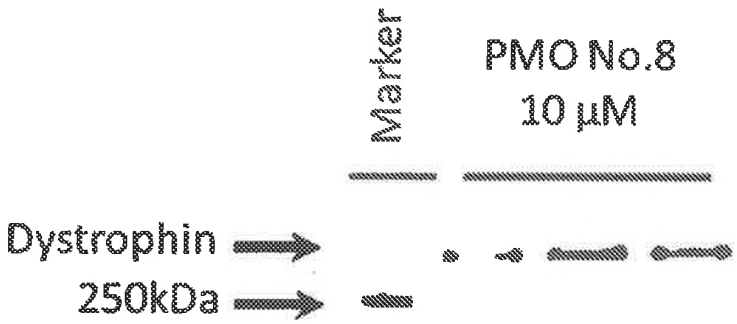

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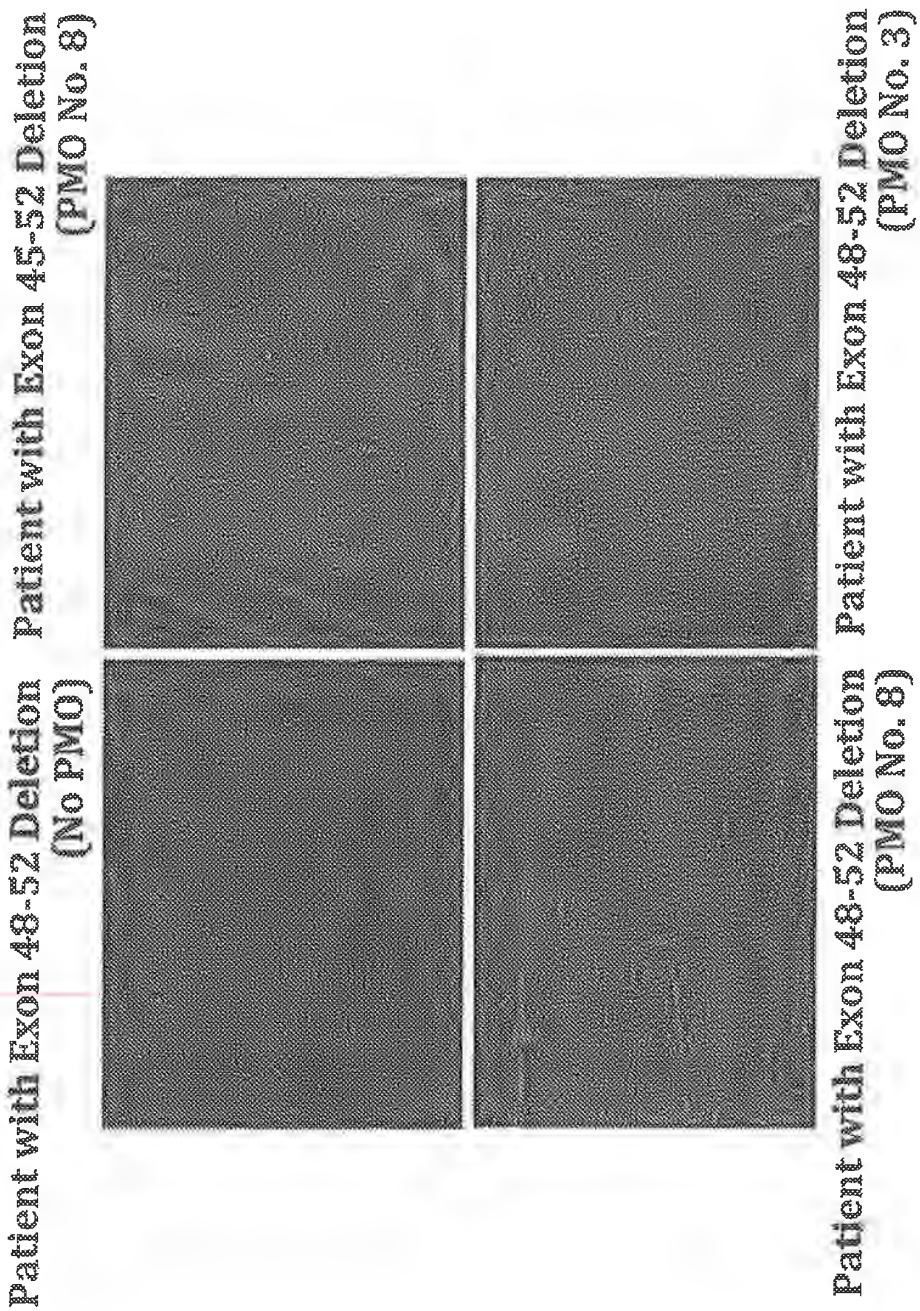

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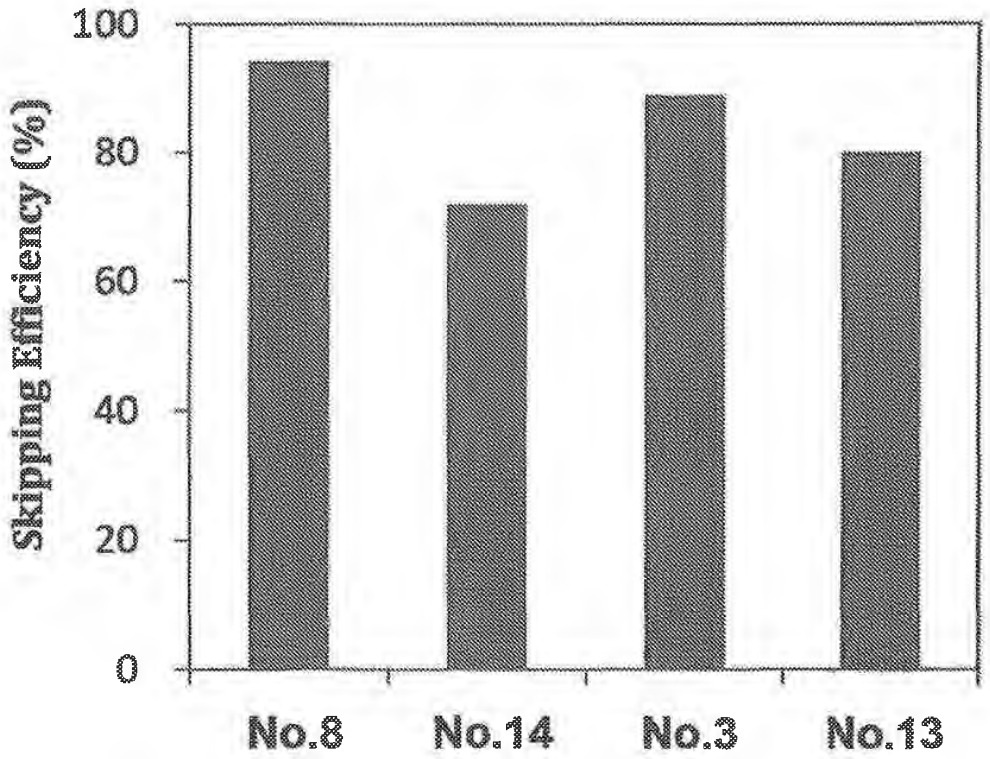
Figure 8



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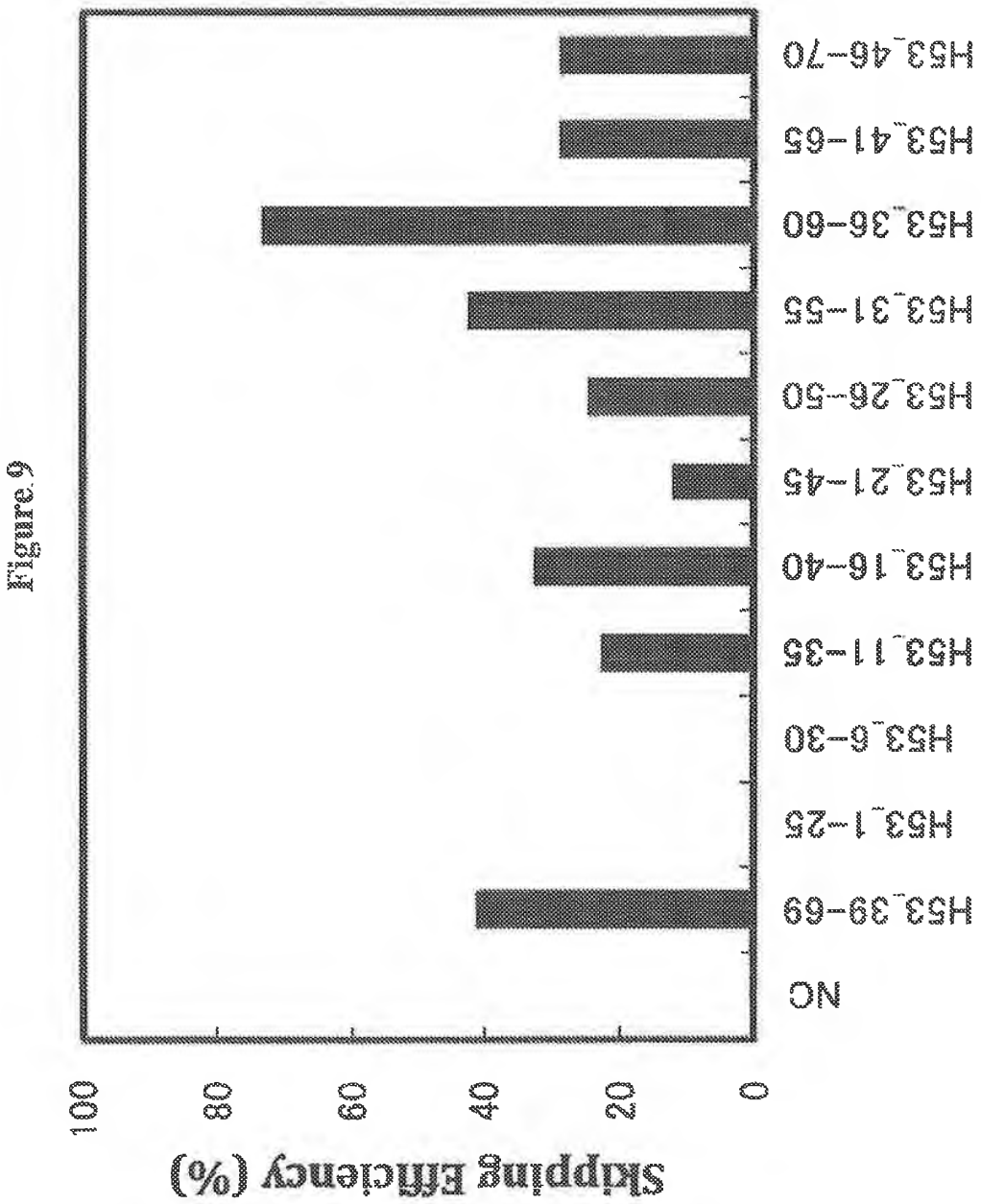

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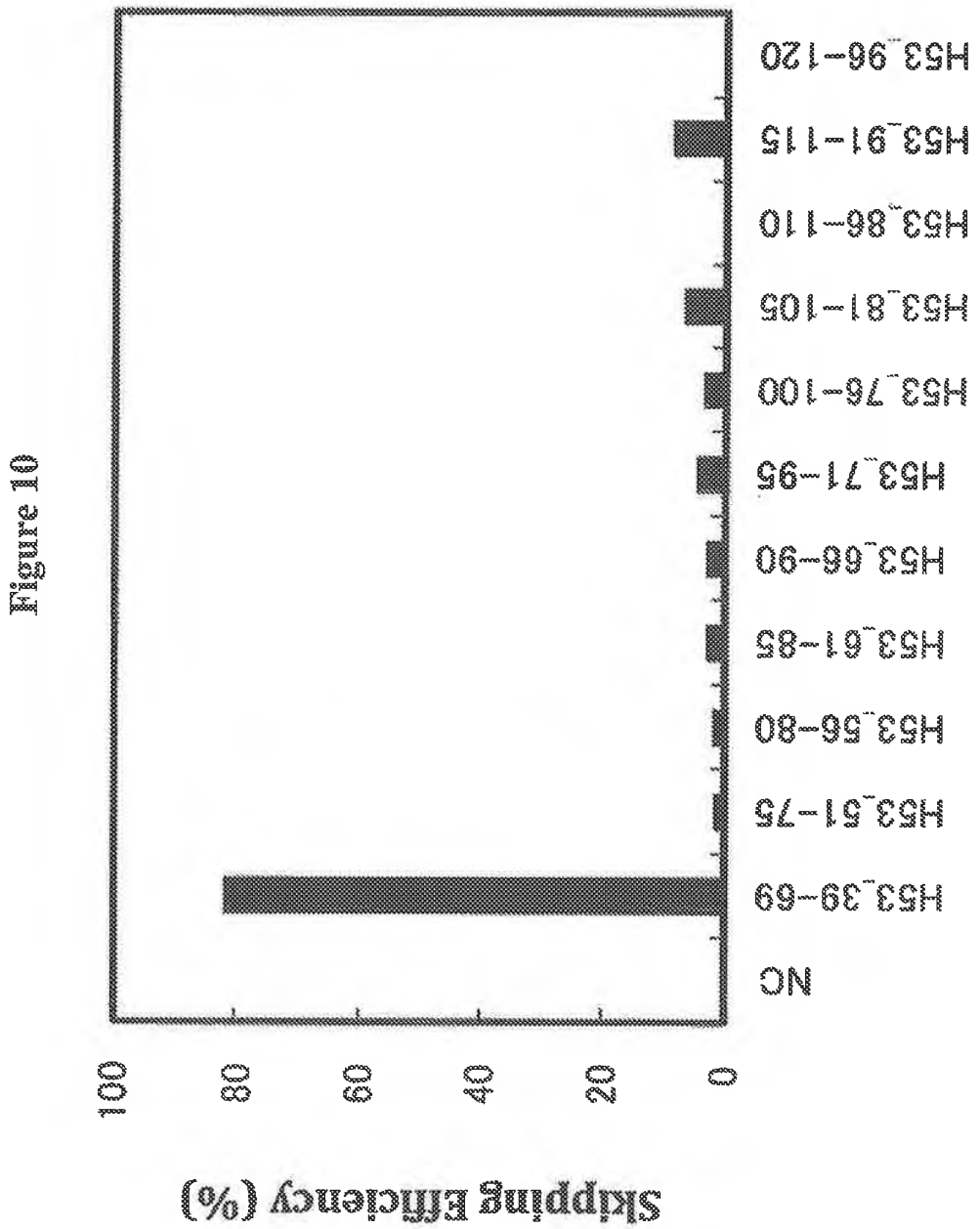

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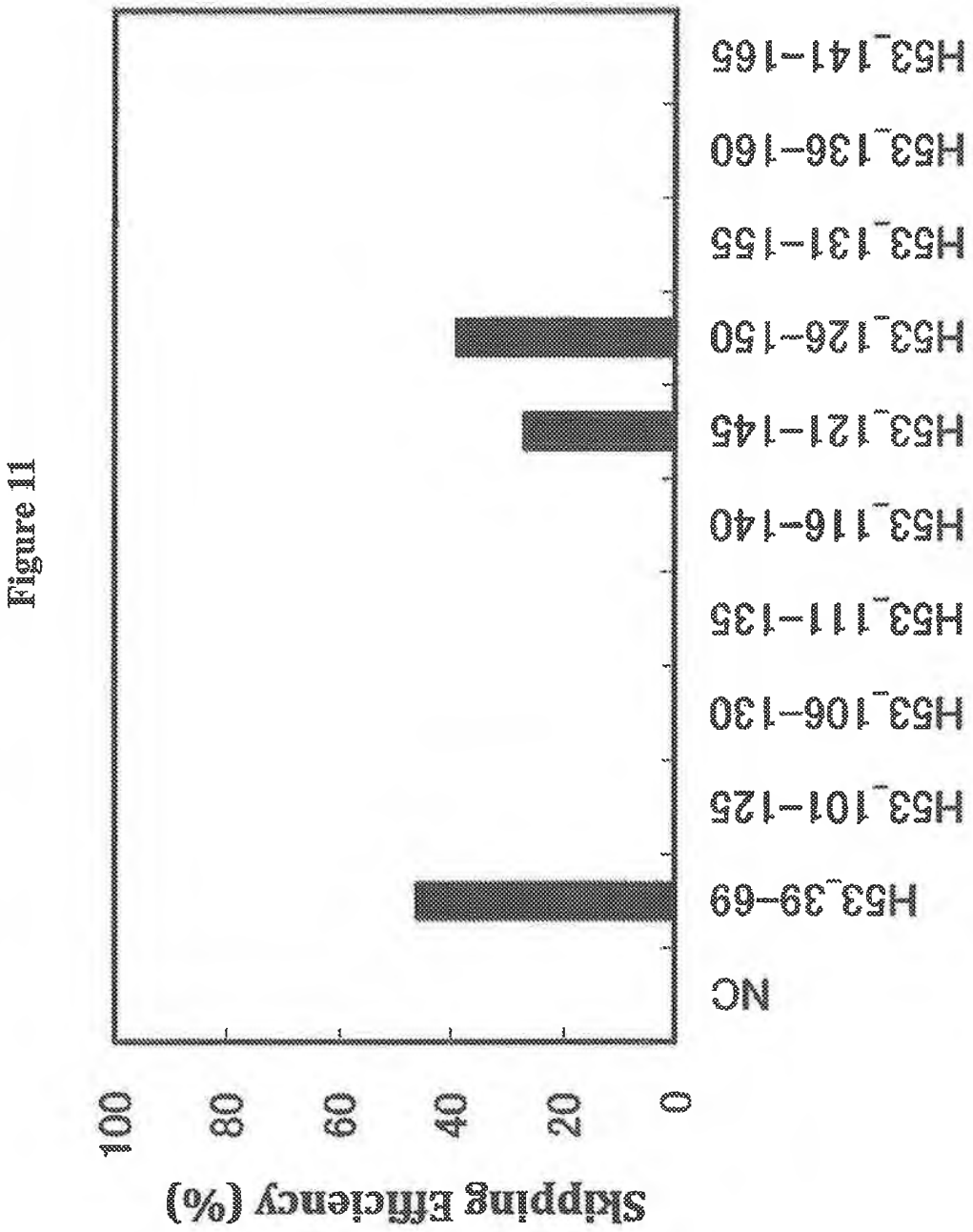

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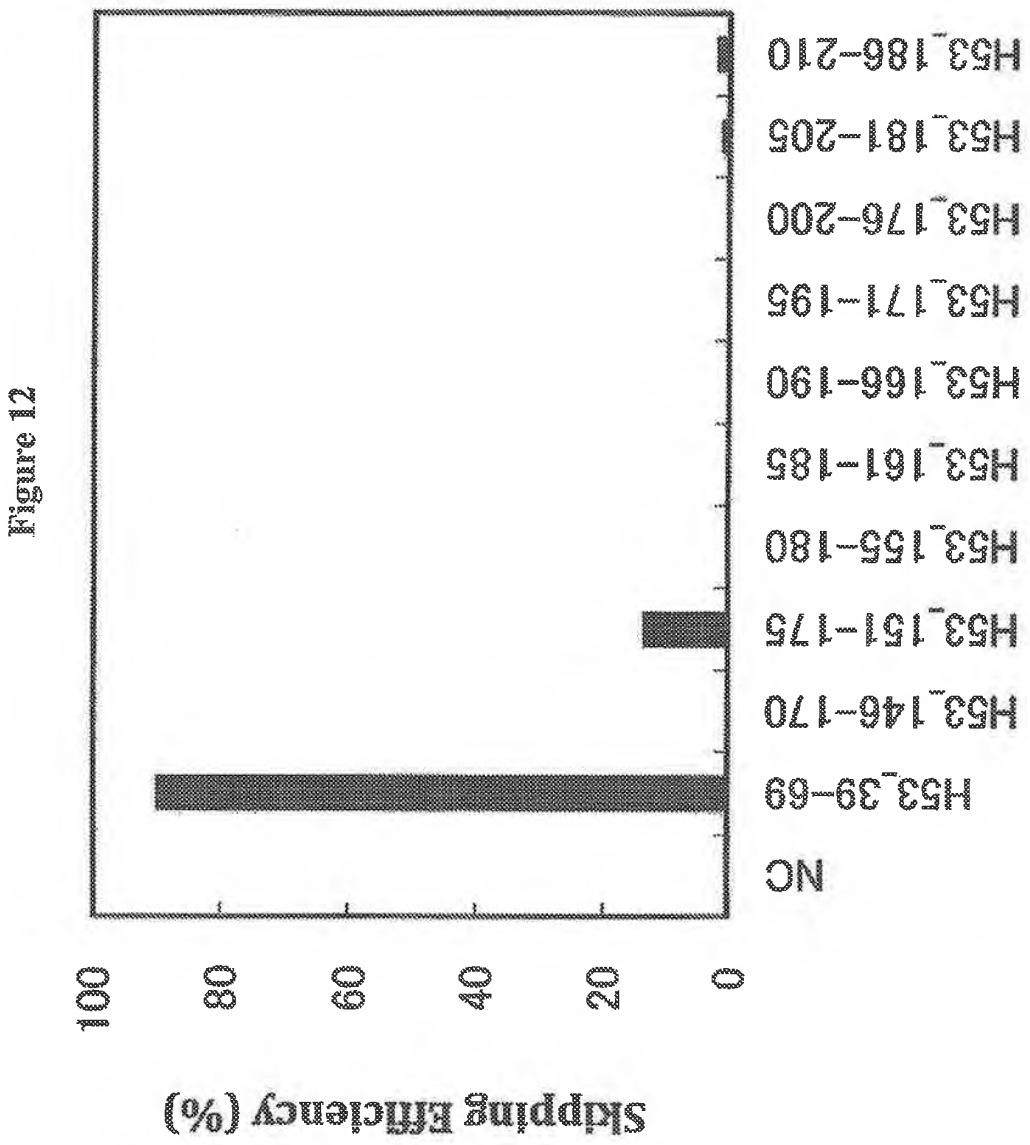

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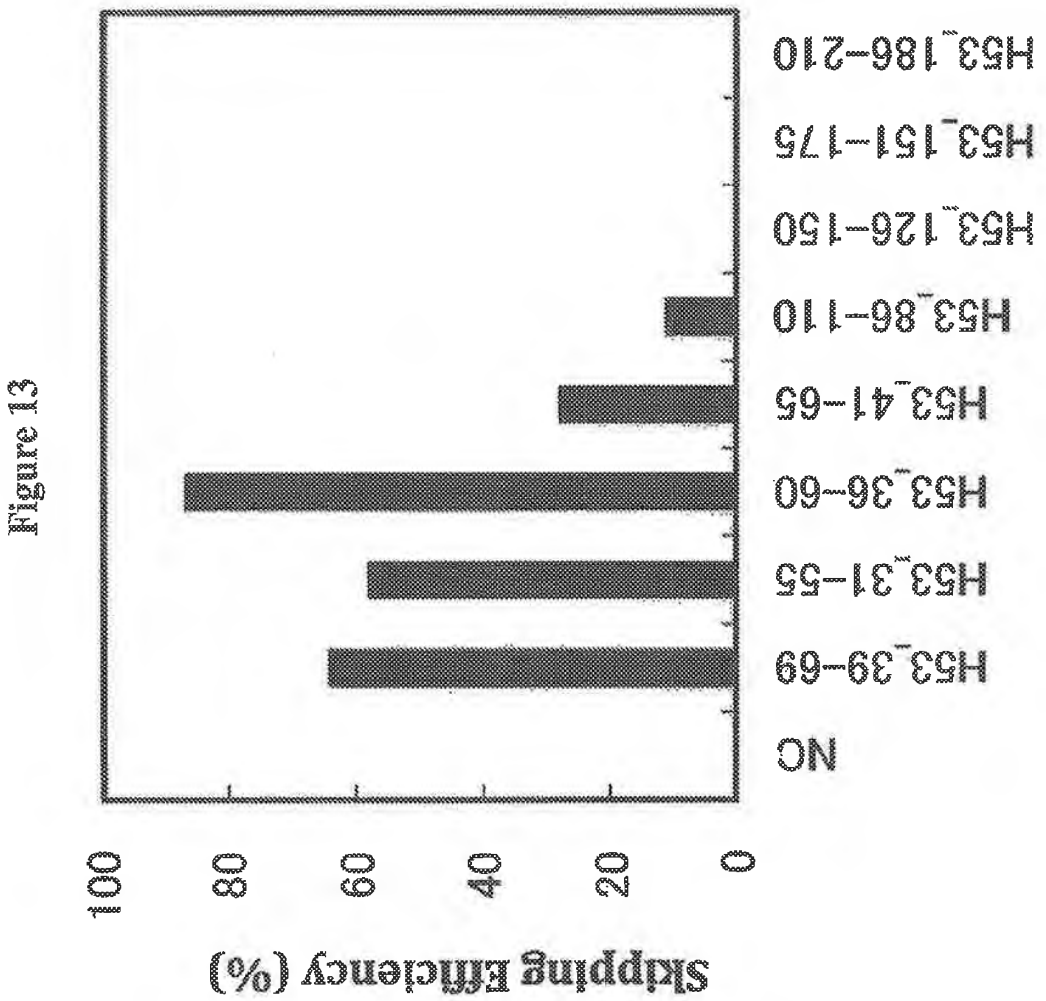

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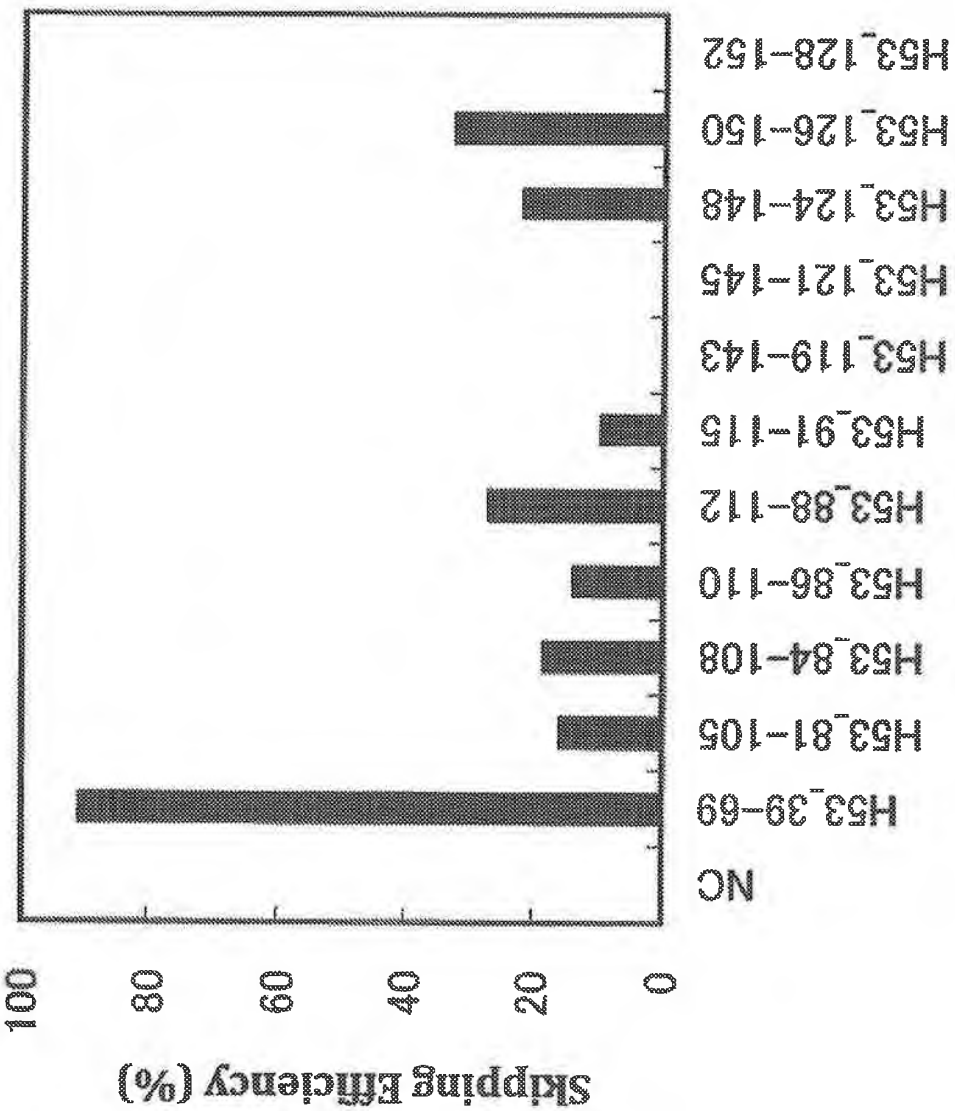

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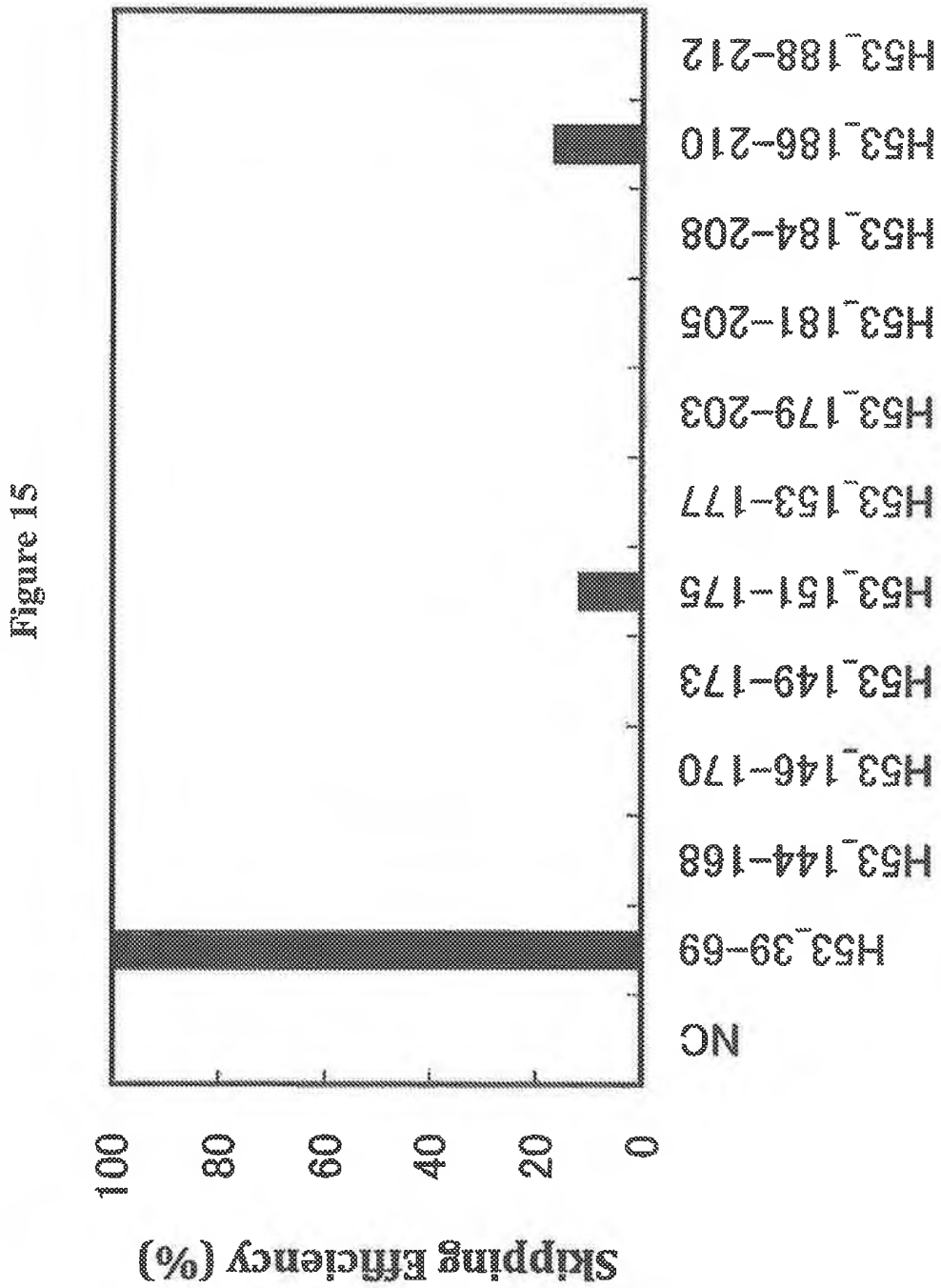

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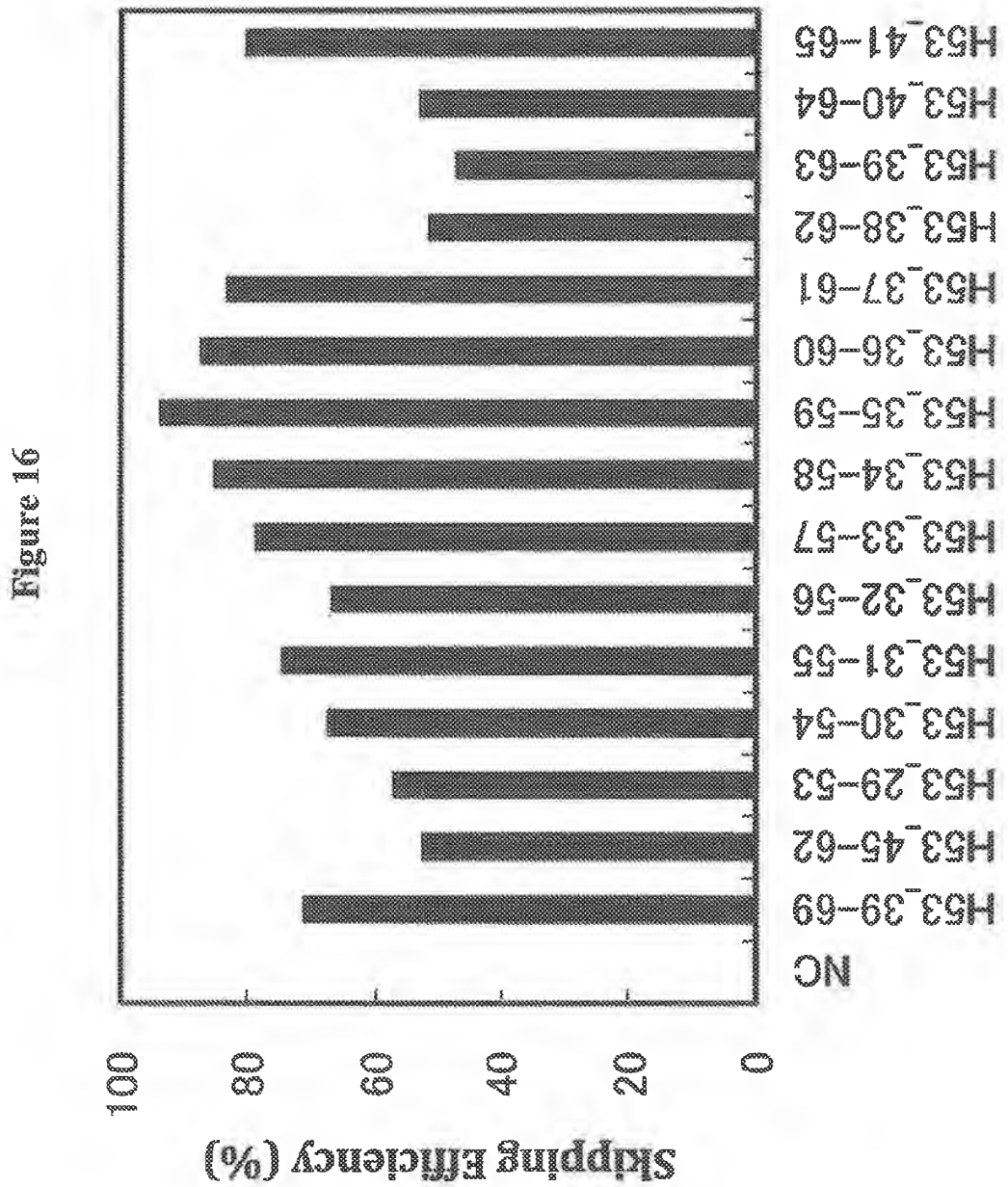

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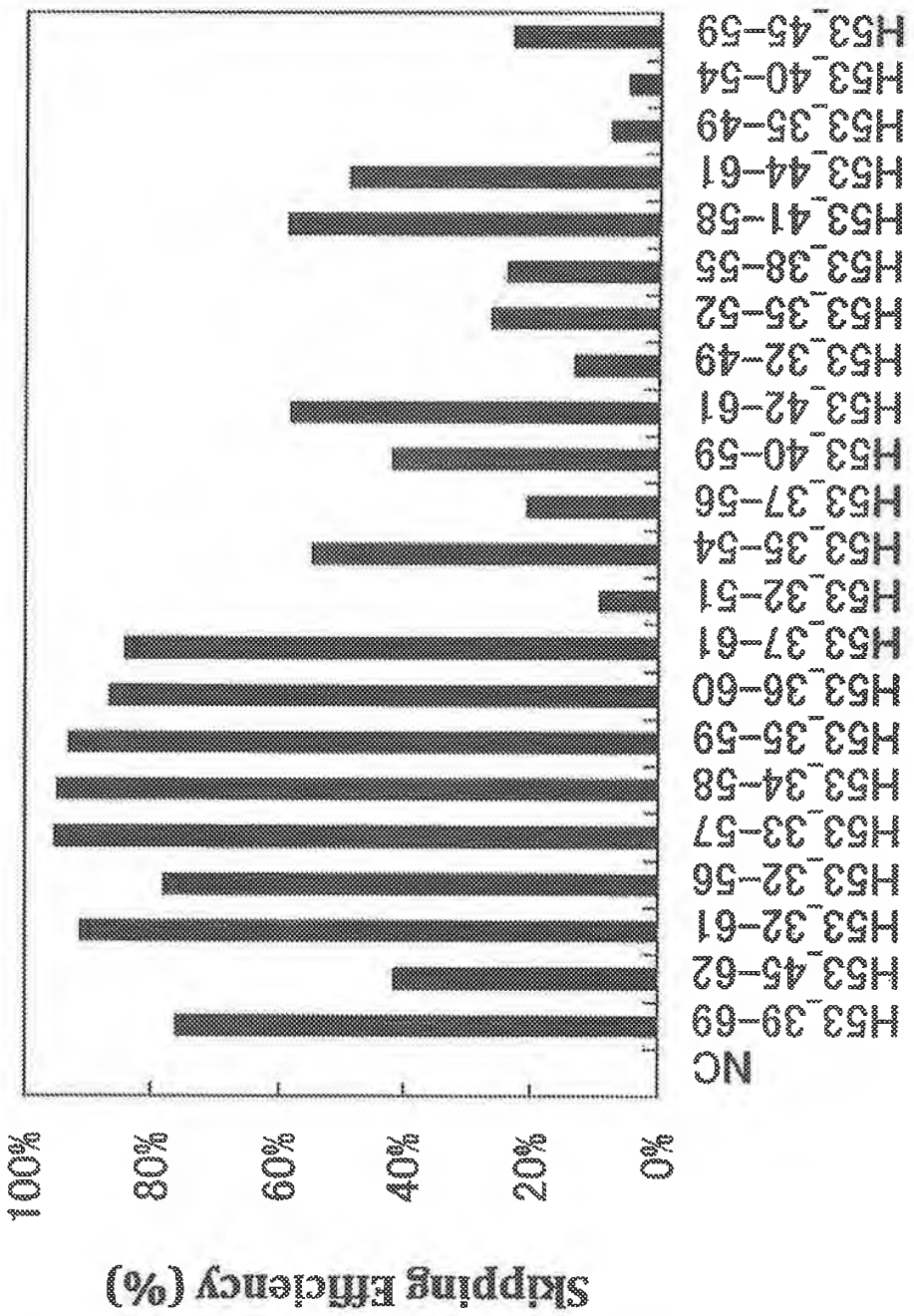

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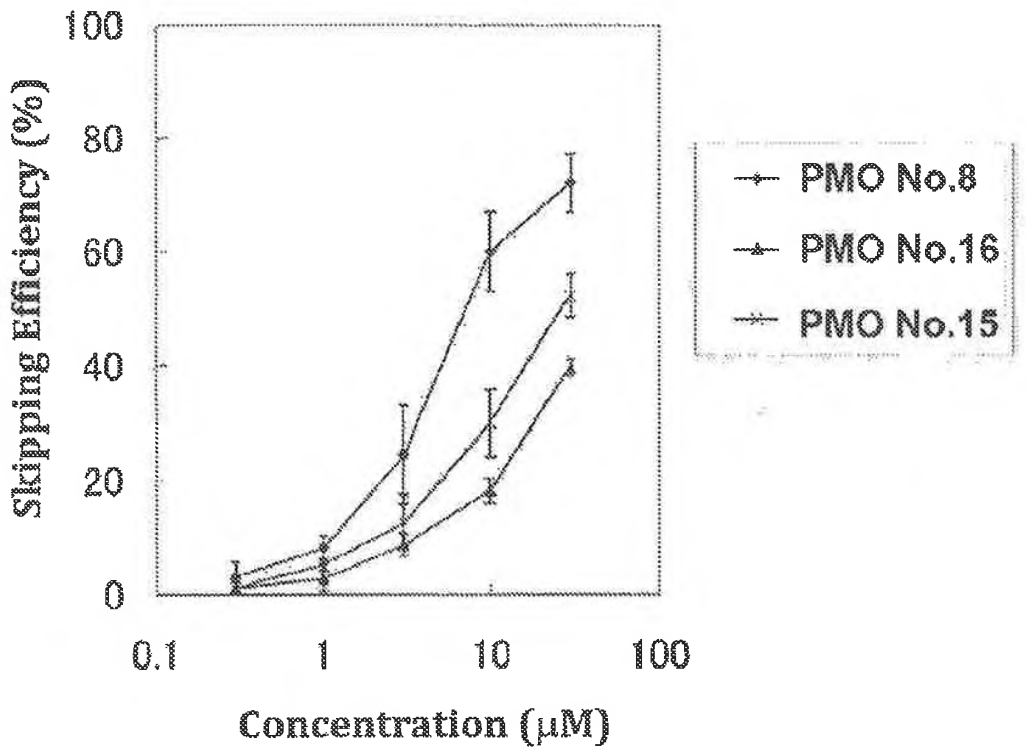

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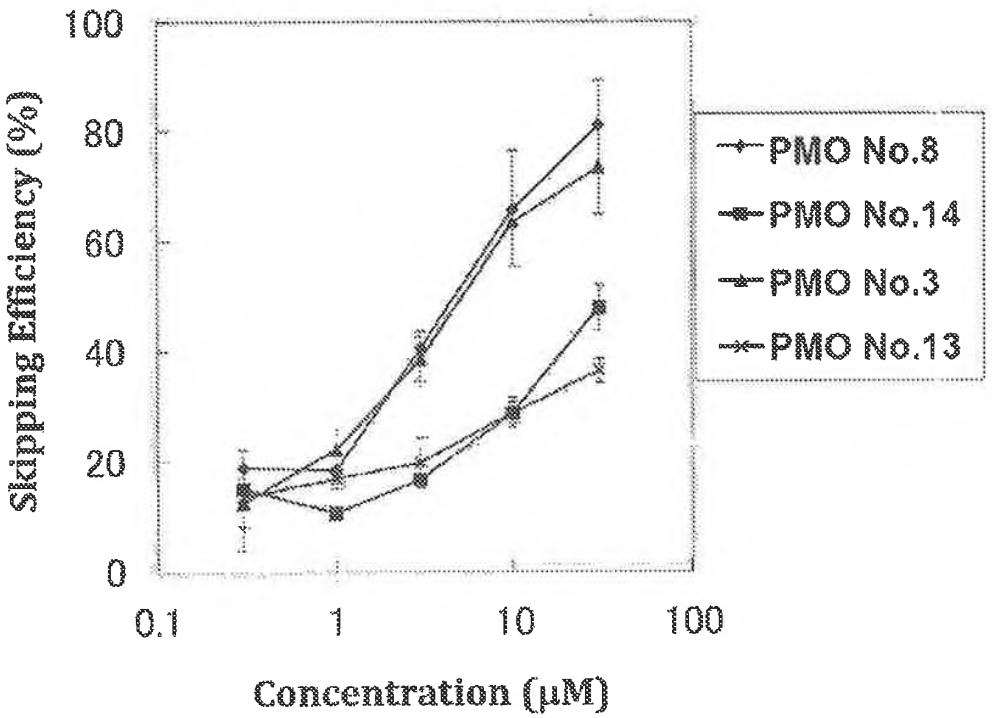

Figure 19

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ANTISENSE NUCLEIC ACIDS

CROSS REFERENCE TO RELATED APPLICATIONS

This is a Continuation of copending application Ser. No. 13/819,520, filed Apr. 10, 2013, which is a PCT National Stage of PCT/JP2011/070318 filed Aug. 31, 2011, which claims priority to JP Application No, 2010-196032 filed Sep. 1, 2010.

SEQUENCE LISTING

A Sequence Listing containing SEQ ID NO: 1-123 is incorporated herein by reference.

TECHNICAL FIELD

The present invention relates to an antisense oligomer which causes skipping of exon 53 in the human dystrophin gene, and a pharmaceutical composition comprising the oligomer.

BACKGROUND ART

Duchenne muscular dystrophy (DMD) is the most frequent form of hereditary progressive muscular dystrophy that affects one in about 3,500 newborn boys. Although the motor functions are rarely different from healthy humans in infancy and childhood, muscle weakness is observed in children from around 4 to 5 years old. Then, muscle weakness progresses to the loss of ambulation by about 12 years old and death due to cardiac or respiratory insufficiency in the twenties. DMD is such a severe disorder. At present, there is no effective therapy for DMD available, and it has been strongly desired to develop a novel therapeutic agent.

DMD is known to be caused by a mutation in the dystrophin gene. The dystrophin gene is located on X chromosome and is a huge gene consisting of 2.2 million DNA nucleotide pairs. DNA is transcribed into mRNA precursors, and introns are removed by splicing to synthesize mRNA in which 79 exons are joined together. This mRNA is translated into 3,685 amino acids to produce the dystrophin protein. The dystrophin protein is associated with the maintenance of membrane stability in muscle cells and necessary to make muscle cells less fragile. The dystrophin gene from patients with DMD contains a mutation and hence, the dystrophin protein, which is functional in muscle cells, is rarely expressed. Therefore, the structure of muscle cells cannot be maintained in the body of the patients with DMD, leading to a large influx of calcium ions into muscle cells. Consequently, an inflammation-like response occurs to promote fibrosis so that muscle cells can be regenerated only with difficulty.

Becker muscular dystrophy (BMD) is also caused by a mutation in the dystrophin gene. The symptoms involve muscle weakness accompanied by atrophy of muscle but are typically mild and slow in the progress of muscle weakness, when compared to DMD. In many cases, its onset is in adulthood. Differences in clinical symptoms between DMD and BMD are considered to reside in whether the reading frame for amino acids on the translation of dystrophin mRNA into the dystrophin protein is disrupted by the mutation or not (Non-Patent Document 1). More specifically, in DMD, the presence of mutation shifts the amino acid reading frame so that the expression of functional dystrophin protein is abolished, whereas in BMD the dystrophin protein that functions, though imperfectly, is produced because the amino acid reading frame is preserved, while a part of the exons are deleted by the mutation.

Exon skipping is expected to serve as a method for treating DMD. This method involves modifying splicing to restore the amino acid reading frame of dystrophin mRNA and induce expression of the dystrophin protein having the function partially restored (Non-Patent Document 2). The amino acid sequence part, which is a target for exon skipping, will be lost. For this reason, the dystrophin protein expressed by this treatment becomes shorter than normal one but since the amino acid reading frame is maintained, the function to stabilize muscle cells is partially retained. Consequently, it is expected that exon skipping will lead DMD to the similar symptoms to that of BMD which is milder. The exon skipping approach has passed the animal tests using mice or dogs and now is currently assessed in clinical trials on human DMD patients.

The skipping of an exon can be induced by binding of antisense nucleic acids targeting either 5' or 3' splice site or both sites, or exon-internal sites. An exon will only be included in the mRNA when both splice sites thereof are recognized by the spliceosome complex. Thus, exon skipping can be induced by targeting the splice sites with antisense nucleic acids. Furthermore, the binding of an SR protein to an exonic splicing enhancer (ESE) is considered necessary for an exon to be recognized by the splicing mechanism. Accordingly, exon skipping can also be induced by targeting ESE.

Since a mutation of the dystrophin gene may vary depending on DMD patients, antisense nucleic acids need to be desined based on the site or type of respective genetic mutation. In the past, antisense nucleic acids that induce exon skipping for all 79 exons were produced by Steve Wilton, et al., University of Western Australia (Non-Patent Document 3), and the antisense nucleic acids which induce exon skipping for 39 exons were produced by Annemieke Aartsma-Rus, et al., Netherlands (Non-Patent Document 4).

It is considered that approximately 8% of all DMD patients may be treated by skipping the 53rd exon (hereinafter referred to as "exon 53"). In recent years, a plurality of research organizations reported on the studies where exon 53 in the dystrophin gene was targeted for exon skipping (Patent Documents 1 to 4; Non-Patent Document 5). However, a technique for skipping exon 53 with a high efficiency has not yet been established.

Patent Document 1: International Publication WO 2006/000057

Patent Document 2: International Publication WO 2004/048570

Patent Document 3: US 2010/0168212

Patent Document 4: International Publication WO 2010/048586

Non-Patent Document 1: Monaco A. P. et al., Genomics 1988; 2: p. 90-95

Non-Patent Document 2: Matsuo M., Brain Dev 1996; 18: p. 167-172

Non-Patent Document 3: Wilton S. D., et al., Molecular Therapy 2007: 15: p. 1288-96

Non-Patent Document 4: Annemieke Aartsma-Rus et al., (2002) Neuromuscular Disorders 12: S71-S77

Non-Patent Document 5: Linda J. Popplewell et al., (2010) Neuromuscular Disorders, vol. 20, no. 2, p. 102-10

DISCLOSURE OF THE INVENTION

Under the foregoing circumstances, antisense oligomers that strongly induce exon 53 skipping in the dystrophin gene and muscular dystrophy therapeutics comprising oligomers thereof have been desired.

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As a result of detailed studies of the structure of the dystrophin gene, the present inventors have found that exon 53 skipping can be induced with a high efficiency by targeting the sequence consisting of the 32nd to the 56th nucleotides from the 5' end of exon 53 in the mRNA precursor (hereinafter referred to as "pre-mRNA") in the dystrophin gene with antisense oligomers. Based on this finding, the present inventors have accomplished the present invention.

That is, the present invention is as follows.

[1] An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences consisting of the 31st to the 53rd, the 31 st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.

[2] The antisense oligomer according to [1] above, which is an oligonucleotide.

[3] The antisense oligomer according to [2] above, wherein the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.

[4] The antisense oligomer according to [3] above, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of OR, R, R'OR, SiH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br and I (wherein R is an alkyl or an aryl and R' is an alkylene).

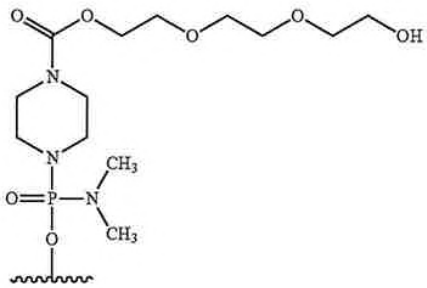
[5] The antisense oligomer according to [3] or [4] above, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond and a boranophosphate bond.

[6] The antisense oligomer according to [1] above, which is a morpholino oligomer.

[7] The antisense oligomer according to [6] above, which is a phosphorodiamidate morpholino oligomer.

[8] The antisense oligomer according to any one of [1] to [7] above, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:

(1)



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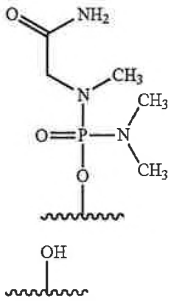
(2)



(3)

[9] The antisense oligomer according to any one of [1] to [8] above, consisting of a nucleotide sequence complementary to the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene.

[10] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 2 to 37.

[11] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 11, 17, 23, 29 and 35.

[12] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by SEQ ID NO: 11 or 35.

[13] A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to any one of [1] to [12] above, or a pharmaceutically acceptable salt or hydrate thereof.

The antisense oligomer of the present invention can induce exon 53 skipping in the human dystrophin gene with a high efficiency. In addition, the symptoms of Duchenne muscular dystrophy can be effectively alleviated by administering the pharmaceutical composition of the present invention.

BRIEF DESCRIPTION OF DRAWINGS

FIG. **1** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cell line (RD cells).

FIG. **2** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human normal tissue-derived fibroblasts (TIG-119 cells) to induce differentiation into muscle cells.

FIG. **3** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human DMD patient-derived fibroblasts (5017 cells) to induce differentiation into muscle cells.

FIG. **4** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. **5** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

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FIG. **6** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. **7** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52 or deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. **8** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. **9** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **10** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **11** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **12** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **13** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **14** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **15** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **16** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **17** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **18** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

FIG. **19** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention is described in detail. The embodiments described below are intended to be presented by way of example merely to describe the invention but not limited only to the following embodiments. The present invention may be implemented in various ways without departing from the gist of the invention.

All of the publications, published patent applications, patents and other patent documents cited in the specification are herein incorporated by reference in their entirety. The specification hereby incorporates by reference the contents of the specification and drawings in the Japanese Patent Application (No. 2010-196032) filed Sep. 1, 2010, from which the priority was claimed.

1. Antisense Oligomer

The present invention provides the antisense oligomer (hereinafter referred to as the "oligomer of the present invention") which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences (hereinafter also referred to as "target sequences") consisting of the 31 st to the 53rd, the 31 st to the 54th, the 31 st to the 55th, the 31 st to the 56th, the 31 st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.

[Exon 53 in Human Dystrophin Gene]

In the present invention, the term "gene" is intended to mean a genomic gene and also include cDNA, mRNA precursor and mRNA. Preferably, the gene is mRNA precursor, i.e., pre-mRNA.

In the human genome, the human dystrophin gene locates at locus Xp21.2. The human dystrophin gene has a size of 3.0 Mbp and is the largest gene among known human genes. However, the coding regions of the human dystrophin gene are only 14 kb, distributed as 79 exons throughout the human dystrophin gene (Roberts, R G., et al., Genomics, 16: 536-538 (1993)). The pre-mRNA, which is the transcript of the human dystrophin gene, undergoes splicing to generate mature mRNA of 14 kb. The nucleotide sequence of human wild-type dystrophin gene is known (GenBank Accession No. NM_004006).

The nucleotide sequence of exon 53 in the human wild-type dystrophin gene is represented by SEQ ID NO: 1.

The oligomer of the present invention is designed to cause skipping of exon 53 in the human dystrophin gene, thereby modifying the protein encoded by DMD type of dystrophin gene into the BMD type of dystrophin protein. Accordingly, exon 53 in the dystrophin gene that is the target of exon skipping by the oligomer of the present invention includes both wild and mutant types.

Specifically, exon 53 mutants of the human dystrophin gene include the polynucleotides defined in (a) or (b) below.

(a) A polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1; and,

(b) A polynucleotide consisting of a nucleotide sequence having at least 90% identity with the nucleotide sequence of SEQ ID NO: 1.

As used herein, the term "polynucleotide" is intended to mean DNA or RNA.

As used herein, the term "polynucleotide that hybridizes under stringent conditions" refers to, for example, a polynucleotide obtained by colony hybridization, plaque hybridization, Southern hybridization or the like, using as a probe all or part of a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of, e.g., SEQ ID NO: 1. The hybridization method which may be used includes methods described in, for example, "Sambrook & Russell, Molecular Cloning: A Laboratory Manual Vol. 3, Cold Spring Harbor, Laboratory Press 2001,"

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"Ausubel, Current Protocols in Molecular Biology, John Wiley & Sons 1987-1997," etc.

As used herein, the term "complementary nucleotide sequence" is not limited only to nucleotide sequences that form Watson-Crick pairs with target nucleotide sequences, but is intended to also include nucleotide sequences which form Wobble base pairs. As used herein, the term Watson-Crick pair refers to a pair of nucleobases in which hydrogen bonds are formed between adenine-thymine, adenine-uracil or guanine-cytosine, and the term Wobble base pair refers to a pair of nucleobases in which hydrogen bonds are formed between guanine-uracil, inosine-uracil, inosine-adenine or inosine-cytosine. As used herein, the term "complementary nucleotide sequence" does not only refers to a nucleotide sequence 100% complementary to the target nucleotide sequence but also refers to a complementary nucleotide sequence that may contain, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target nucleotide sequence.

As used herein, the term "stringent conditions" may be any of low stringent conditions, moderate stringent conditions or high stringent conditions. The term "low stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 32° C. The term "moderate stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 42° C., or 5×SSC, 1% SDS, 50 mM Tris-HCl (pH 7.5), 50% formamide at 42° C. The term "high stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 50° C. or 0.2×SSC, 0.1% SDS at 65° C. Under these conditions, polynucleotides with higher homology are expected to be obtained efficiently at higher temperatures, although multiple factors are involved in hybridization stringency including temperature, probe concentration, probe length, ionic strength, time, salt concentration and others, and those skilled in the art may appropriately select these factors to achieve similar stringency.

When commercially available kits are used for hybridization, for example, an Alkphos Direct Labeling and Detection System (GE Healthcare) may be used. In this case, according to the attached protocol, after cultivation with a labeled probe overnight, the membrane is washed with a primary wash buffer containing 0.1% (w/v) SDS at 55° C., thereby detecting hybridized polynucleotides. Alternatively, in producing a probe based on the entire or part of the nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1, hybridization can be detected with a DIG Nucleic Acid Detection Kit (Roche Diagnostics) when the probe is labeled with digoxigenin (DIG) using a commercially available reagent (e.g., a PCR Labeling Mix (Roche Diagnostics), etc.).

In addition to the polynucleotides described above, other polynucleotides that can be hybridized include polynucleotides having 90% or higher, 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, 98% or higher, 99% or higher, 99.1% or higher, 99.2% or higher, 99.3% or higher, 99.4% or higher, 99.5% or higher, 99.6% or higher, 99.7% or higher, 99.8% or higher or 99.9% or higher identity with the polynucleotide of SEQ ID NO: 1, as calculated by homology search software BLAST using the default parameters.

The identity between nucleotide sequences may be determined using algorithm BLAST (Basic Local Alignment Search Tool) by Karlin and Altschul (Proc. Natl. Acad. Sci. USA 872264-2268, 1990; Proc. Natl. Acad. Sci. USA 90: 5873, 1993). Programs called BLASTN and BLASTX based on the BLAST algorithm have been developed (Altschul S F, et al: J. Mol. Biol. 215: 403, 1990). When a nucleotide sequence is sequenced using BLASTN, the parameters are, for example, score=100 and wordlength=12. When BLAST and Gapped BLAST programs are used, the default parameters for each program are employed.

Examples of the nucleotide sequences complementary to the sequences consisting of the 31 st to the 53rd, the 31 st to the 54th, the 31 st to the 55th, the 31 st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th and the 36th to the 58th nucleotides, from the 5' end of exon 53.

TABLE 1

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
31-53	5'-CCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 2
31-54	5'-TCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 3
31-55	5'-CTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 4
31-56	5'-CCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 5
31-57	3'-GCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO; 6
31-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 7
32-53	5'-CCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 8
32-54	5'-TCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 9
32-55	5'-CTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 10
32-56	5'CCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 11
32-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 12

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TABLE 1-continued

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
32-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 13
33-53	5'-CCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 14
33-54	5'-TCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 15
33-55	5'-CTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 16
33-56	5'-CCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 17
33-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 18
33-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 19
34-53	5'-CCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 20
34-54	5'TCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 21
34-55	5'-CTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 22
34-56	5'-CCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 23
34-57	5'-GCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 24
34-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 25
35-53	5'-CCGGTTCTGAAGGTGTTCT-'3	SEQ ID NO: 26
35-54	5'-TCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 27
35-55	5'-CTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 28
35-56	5'-CCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 29
35-57	5'-GCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 30
35-58	5'TGCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 31
36-53	5'-CCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 32
36-54	5'-TCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 33
36-55	5'-CTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 34
36-56	5'-CCTCCGGTTCTCGAAGGTGTTC-3'	SEQ ID NO: 35
36-57	5'-GCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 36
36-58	5'-TGCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 37

It is preferred that the oligomer of the present invention consists of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th, the 33rd to the 56th, the 34th to the 56th, the 35th to the 56th or the 36th to the 56th nucleotides (e.g., SEQ ID NO: 11, SEQ ID NO: 17, SEQ ID NO: 23, SEQ ID NO: 29 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

Preferably, the oligomer of the present invention consists of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides (e.g., SEQ ID NO: 11 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

The term "cause skipping of the 53rd exon in the human dystrophin gene" is intended to mean that by binding of the oligomer of the present invention to the site corresponding to exon 53 of the transcript (e.g., pre-mRNA) of the human dystrophin gene, for example, the nucleotide sequence corresponding to the 5' end of exon 54 is spliced at the 3' side of the nucleotide sequence corresponding to the 3' end of exon 51 in DMD patients with deletion of, exon 52 when the transcript undergoes splicing, thus resulting in formation of mature mRNA which is free of codon frame shift.

Accordingly, it is not required for the oligomer of the present invention to have a nucleotide sequence 100% complementary to the target sequence, as far as it causes exon 53 skipping in the human dystrophin gene. The oligomer of the present invention may include, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target sequence.

Herein, the term "binding" described above is intended to mean that when the oligomer of the present invention is mixed with the transcript of human dystrophin gene, both are hybridized under physiological conditions to form a double strand nucleic acid. The term "under physiological conditions" refers to conditions set to mimic the in vivo environment in terms of pH, salt composition and tempera-

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ture. The conditions are, for example, 25 to 40° C., preferably 37° C., pH 5 to 8, preferably pH 7.4 and 150 mM of sodium chloride concentration.

Whether the skipping of exon 53 in the human dystrophin gene is caused or not can be confirmed by introducing the oligomer of the present invention into a dystrophin expression cell (e.g., human rhabdomyosarcoma cells), amplifying the region surrounding exon 53 of mRNA of the human dystrophin gene from the total RNA of the dystrophin expression cell by RT-PCR and performing nested PCR or sequence analysis on the PCR amplified product.

The skipping efficiency can be determined as follows. The mRNA for the human dystrophin gene is collected from test cells; in the mRNA, the polynucleotide level "A" of the band where exon 53 is skipped and the polynucleotide level "B" of the band where exon 53 is not skipped are measured. Using these measurement values of "A" and "B," the efficiency is calculated by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A+B) \times 100$$

The oligomer of the present invention includes, for example, an oligonucleotide, morpholino oligomer or peptide nucleic acid (PNA), having a length of 18 to 28 nucleotides. The length is preferably from 21 to 25 nucleotides and morpholino oligomers are preferred.

The oligonucleotide described above (hereinafter referred to as "the oligonucleotide of the present invention") is the oligomer of the present invention composed of nucleotides as constituent units. Such nucleotides may be any of ribonucleotides, deoxyribonucleotides and modified nucleotides.

The modified nucleotide refers to one having fully or partly modified nucleobases, sugar moieties and/or phosphate-binding regions, which constitute the ribonucleotide or deoxyribonucleotide.

The nucleobase includes, for example, adenine, guanine, hypoxanthine, cytosine, thymine, uracil, and modified bases thereof. Examples of such modified nucleobases include, but not limited to, pseudouracil, 3-methyluracil, dihydrouracil, 5-alkylcytosines (e.g., 5-methylcytosine), 5-alkyluracils (e.g., 5-ethyluracil), 5-halouracils (5-bromouracil), 6-azapyrimidine, 6-alkylpyrimidines (6-methyluracil), 2-thiouracil, 4-thiouracil, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5'-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, 1-methyladenine, 1-methylhypoxanthine, 2,2-dimethylguanine, 3-methylcytosine, 2-methyladenine, 2-methylguanine, N6-methyladenine, 7-methylguanine, 5-methoxyaminomethyl-2-thiouracil, 5-methylaminomethyluracil, 5-methylcarbonylmethyluracil, 5-methyloxyuracil, 5-methyl-2-thiouracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid, 2-thiocytosine, purine, 2,6-diaminopurine, 2-aminopurine, isoguanine, indole, imidazole, xanthine, etc.

Modification of the sugar moiety may include, for example, modifications at the 2'-position of ribose and modifications of the other positions of the sugar. The modification at the 2'-position of ribose includes replacement of the 2'-OH of ribose with OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br or I, wherein R represents an alkyl or an aryl and R' represents an alkylene.

The modification for the other positions of the sugar includes, for example, replacement of O at the 4' position of ribose or deoxyribose with S, bridging between 2' and 4' positions of the sugar, e.g., LNA (locked nucleic acid) or ENA (2'-O,4'-C-ethylene-bridged nucleic acids), but is not limited thereto.

A modification of the phosphate-binding region includes, for example, a modification of replacing phosphodiester bond with phosphorothioate bond, phosphorodithioate bond, alkyl phosphonate bond, phosphoroamidate bond or boranophosphate bond (Enya et al: Bioorganic & Medicinal Chemistry, 2008, 18, 9154-9160) (cf., e.g., Japan Domestic Re-Publications of PCT Application Nos. 2006/129594 and 2006/038608).

The alkyl is preferably a straight or branched alkyl having 1 to 6 carbon atoms. Specific examples include methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, n-pentyl, isopentyl, neopentyl, tert-pentyl, n-hexyl and isohexyl. The alkyl may optionally be substituted. Examples of such substituents are a halogen, an alkoxy, cyano and nitro. The alkyl may be substituted with 1 to 3 substituents.

The cycloalkyl is preferably a cycloalkyl having 5 to 12 carbon atoms. Specific examples include cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, cyclodecyl and cyclododecyl.

The halogen includes fluorine, chlorine, bromine and iodine.

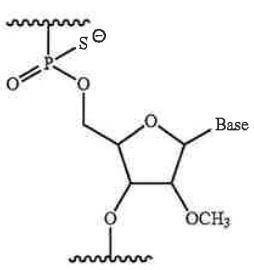
The alkoxy is a straight or branched alkoxy having 1 to 6 carbon atoms such as methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, isobutoxy, sec-butoxy, tert-butoxy, n-pentyloxy, isopentyloxy, n-hexyloxy, isohexyloxy, etc. Among others, an alkoxy having 1 to 3 carbon atoms is preferred.

The aryl is preferably an aryl having 6 to 10 carbon atoms. Specific examples include phenyl, α-naphthyl and β-naphthyl. Among others, phenyl is preferred. The aryl may optionally be substituted. Examples of such substituents are an alkyl, a halogen, an alkoxy, cyano and nitro. The aryl may be substituted with one to three of such substituents.

The alkylene is preferably a straight or branched alkylene having 1 to 6 carbon atoms. Specific examples include methylene, ethylene, trimethylene, tetramethylene, pentamethylene, hexamethylene, 2-(ethyl)trimethylene and 1-(methyl)tetramethylene.

The acyl includes a straight or branched alkanoyl or aroyl. Examples of the alkanoyl include formyl, acetyl, 2-methylacetyl, 2,2-dimethylacetyl, propionyl, butyryl, isobutyryl, pentanoyl, 2,2-dimethylpropionyl, hexanoyl, etc. Examples of the aroyl include benzoyl, toluoyl and naphthoyl. The aroyl may optionally be substituted at substitutable positions and may be substituted with an alkyl(s).

Preferably, the oligonucleotide of the present invention is the oligomer of the present invention containing a constituent unit represented by general formula below wherein the —OH group at position 2' of ribose is substituted with methoxy and the phosphate-binding region is a phosphorothioate bond:



wherein Base represents a nucleobase.

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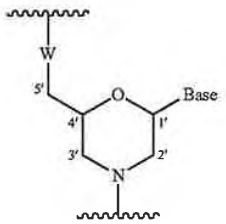
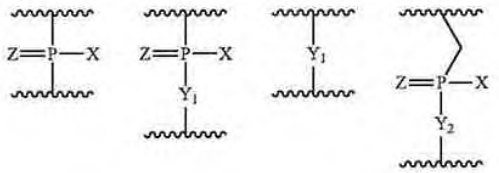
The oligonucleotide of the present invention may be easily synthesized using various automated synthesizer (e.g., AKTA oligopilot plus 10/100 (GE Healthcare)). Alternatively, the synthesis may also be entrusted to a third-party organization (e.g., Promega Inc., or Takara Co.), etc.

The morpholino oligomer of the present invention is the oligomer of the present invention comprising the constituent unit represented by general formula below:



wherein Base has the same significance as defined above, and,

W represents a group shown by any one of the following groups:



wherein

X represents $—CH_2R^1$, $—O—CH_2R^1$, $—S—CH_2R^1$, $—NR_2R^3$ or F;

R^1 represents H or an alkyl;

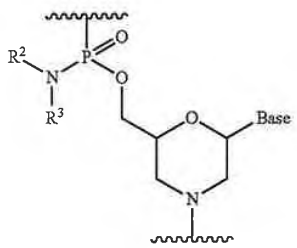
R^2 and R^3, which may be the same or different, each represents H, an alkyl, a cycloalkyl or an aryl;

Y_1 represents O, S, CH_2 or NR^1;

Y_2 represents O, S or NR^1;

Z represents O or S.

Preferably, the morpholino oligomer is an oligomer comprising a constituent unit represented by general formula below (phosphorodiamidate morpholino oligomer (hereinafter referred to as "PMO")).



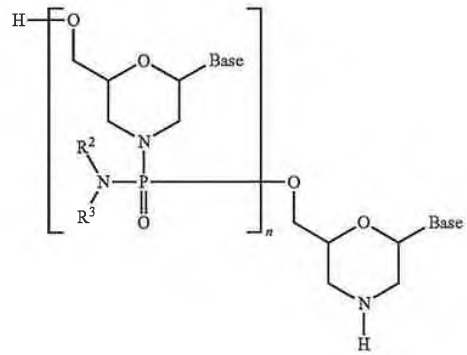
wherein Base, R^2 and R^3 have the same significance as defined above.

The morpholino oligomer may be produced in accordance with, e.g., WO 1991/009033 or WO 2009/064471. In particular, PMO can be produced by the procedure described in WO 2009/064471 or produced by the process shown below.

[Method for Producing PMO]

An embodiment of PMO is, for example, the compound represented by general formula (I) below (hereinafter PMO (I)).

(I)



wherein

Base, R^2 and R^3 have the same significance as defined above; and,

n is a given integer of 1 to 99, preferably a given integer of 18 to 28.

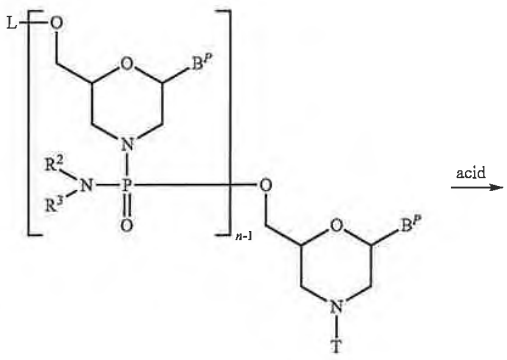
PMO (I) can be produced in accordance with a known method, for example, can be produced by performing the procedures in the following steps.

The compounds and reagents used in the steps below are not particularly limited so long as they are commonly used to prepare PMO.

Also, the following steps can all be carried out by the liquid phase method or the solid phase method (using manuals or commercially available solid phase automated synthesizers). In producing PMO by the solid phase method, it is desired to use automated synthesizers in view of simple operation procedures and accurate synthesis.

(1) Step A:

The compound represented by general formula (II) below (hereinafter referred to as Compound (II)) is reacted with an acid to prepare the compound represented by general formula (III) below (hereinafter referred to as Compound (III)):



(II)

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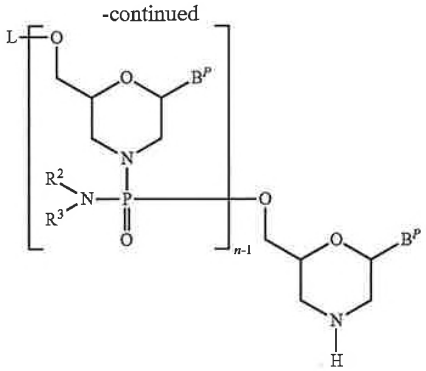


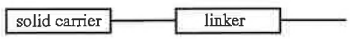
(III)

wherein n, R^2 and R^3 have the same significance as defined above;

each B^P independently represents a nucleobase which may optionally be protected;

T represents trityl, monomethoxytrityl or dimethoxytrityl; and,

L represents hydrogen, an acyl or a group represented by general formula (TV) below (hereinafter referred to as group (IV)).



(IV)

The "nucleobase" for B^P includes the same "nucleobase" as in Base, provided that the amino or hydroxy group in the nucleobase shown by B^P may be protected.

Such protective group for amino is not particularly limited so long as it is used as a protective group for nucleic acids. Specific examples include benzoyl, 4-methoxybenzoyl, acetyl, propionyl, butyryl, isobutyryl, phenylacetyl, phenoxyacetyl, 4-tert-butylphenoxyacetyl, 4-isopropylphenoxyacetyl and (dimethylamino)methylene. Specific examples of the protective group for the hydroxy group include 2-cyanoethyl, 4-nitrophenethyl, phenylsulfonylethyl, methylsulfonylethyl and trimethylsilylethyl, and phenyl, which may be substituted by 1 to 5 electron-withdrawing group at optional substitutable positions, diphenylcarbamoyl, dimethylcarbamoyl, diethylcarbamoyl, methylphenylcarbamoyl, 1-pyrolidinylcarbamoyl, morpholinocarbamoyl, 4-(tert-butylcarboxy)benzyl, 4-[(dimethylamino)carboxy]benzyl and 4-(phenylcarboxy)benzyl, (cf., e.g., WO 2009/064471).

The "solid carrier" is not particularly limited so long as it is a carrier usable for the solid phase reaction of nucleic acids. It is desired for the solid carrier to have the following properties: e.g., (i) it is sparingly soluble in reagents that can be used for the synthesis of morpholino nucleic acid derivatives (e.g., dichloromethane, acetonitrile, tetrazole, N-methylimidazole, pyridine, acetic anhydride, lutidine, trifluoroacetic acid); (ii) it is chemically stable to the reagents usable for the synthesis of morpholino nucleic acid derivatives; (iii) it can be chemically modified; (iv) it can be charged with desired morpholino nucleic acid derivatives; (v) it has a strength sufficient to withstand high pressure through treatments; and (vi) it has a uniform particle diameter range and distribution. Specifically, swellable polystyrene (e.g., aminomethyl polystyrene resin 1% dibenzylbenzene crosslinked (200-400 mesh) (2.4-3.0 mmol/g) (manufactured by Tokyo Chemical Industry), Aminomethylated Polystyrene Resin.HCl [dibenzylbenzene 1%, 100-200 mesh] (manufactured by Peptide Institute, Inc.)), non-swellable polystyrene (e.g., Primer Support (manufactured by GE Healthcare)), PEG chain-attached polystyrene (e.g., NH_2—PEG resin (manufactured by Watanabe Chemical Co.), TentaGel resin), controlled pore glass (controlled pore glass; CPG) (manufactured by, e.g., CPG), oxalyl-controlled pore glass (cf., e.g., Alul et al., Nucleic Acids Research, Vol. 19, 1527 (1991)), TentaGel support-aminopolyethylene glycol-derivatized support (e.g., Wright et al., cf., Tetrahedron Letters, Vol. 34, 3373 (1993)), and a copolymer of Poros-polystyrene/divinylbenzene.

A "linker" which can be used is a known linker generally used to connect nucleic acids or morpholino nucleic acid derivatives. Examples include 3-aminopropyl, succinyl, 2,2'-diethanolsulfonyl and a long chain alkyl amino (LCAA).

This step can be performed by reacting Compound (11) with an acid.

The "acid" which can be used in this step includes, for example, trifluoroacetic acid, dichloroacetic acid and trichloroacetic acid. The acid used is appropriately in a range of, for example, 0.1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (II), preferably in a range of 1 mol equivalent to 100 mol equivalents based on 1 mol of Compound (II).

An organic amine can be used in combination with the acid described above. The organic amine is not particularly limited and includes, for example, triethylamine. The amount of the organic amine used is appropriately in a range of, e.g., 0.01 mol equivalent to 10 mol equivalents, and preferably in a range of 0.1 mol equivalent to 2 mol equivalents, based on 1 mol of the acid.

When a salt or mixture of the acid and the organic amine is used in this step, the salt or mixture includes, for example, a salt or mixture of trifluoroacetic acid and triethylamine, and more specifically, a mixture of 1 equivalent of triethylamine and 2 equivalents of trifluoroacetic acid.

The acid which can be used in this step may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

The reaction temperature in the reaction described above is preferably in a range of, e.g., 10° C. to 50° C., more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C.

The reaction time may vary depending upon kind of the acid used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

After completion of this step, a base may be added, if necessary, to neutralize the acid remained in the system. The "base" is not particularly limited and includes, for example, diisopropylamine. The base may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% (v/v) to 30% (v/v).

The solvent used in this step is not particularly limited so long as it is inert to the reaction, and includes dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, and a mixture thereof. The reaction temperature is preferably in a range of, e.g., 10° C. to 50° C., more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C.

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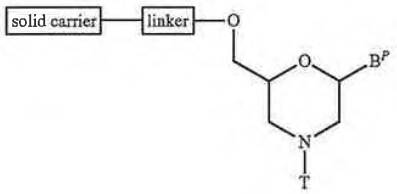
The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

In Compound (II), the compound of general formula (IIa) below (hereinafter Compound (IIa)), wherein n is 1 and L is a group (IV), can be produced by the following procedure.

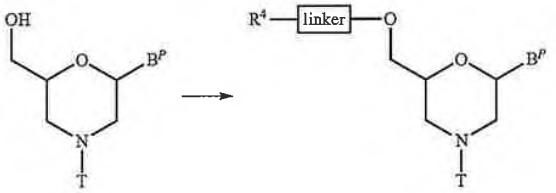


(IIa)

wherein B^P, T, linker and solid carrier have the same significance as defined above.

Step 1

The compound represented by general formula (V) below is reacted with an acylating agent to prepare the compound represented by general formula (VI) below (hereinafter referred to as Compound (VI)).



(V) (VI)

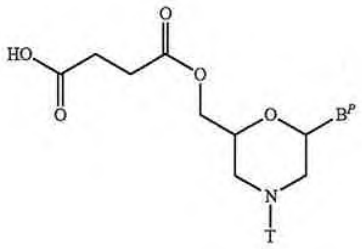
wherein B^P, T and linker have the same significance as defined above; and,

R^4 represents hydroxy, a halogen or amino.

This step can be carried out by known procedures for introducing linkers, using Compound (V) as the starting material.

In particular, the compound represented by general formula (VIa) below can be produced by performing the method known as esterification, using Compound (V) and succinic anhydride.

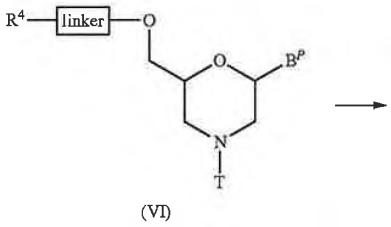
(VIa)



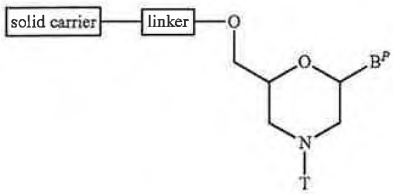
wherein B^P and T have the same significance as defined above.

Step 2

Compound (VI) is reacted with a solid career by a condensing agent to prepare Compound (IIa).



(VI)



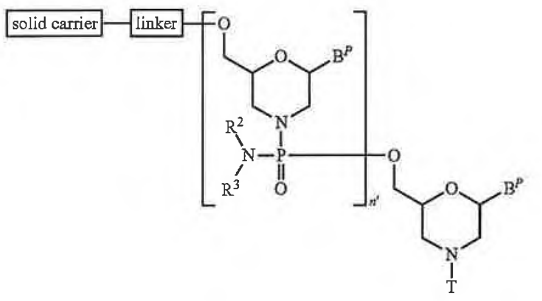
(IIa)

wherein B^P, R^4, T, linker and solid carrier have the same significance as defined above.

This step can be performed using Compound (VI) and a solid carrier in accordance with a process known as condensation reaction.

In Compound (II), the compound represented by general formula (IIa2) below wherein n is 2 to 99 and L is a group represented by general formula (IV) can be produced by using Compound (IIa) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

(IIa2)



wherein B^P, R^2, R^3, T, linker and solid carrier have the same significance as defined above; and,

n' represents 1 to 98.

In Compound (II), the compound of general formula (IIb) below wherein n is 1 and L is hydrogen can be produced by the procedure described in, e.g., WO 1991/009033.

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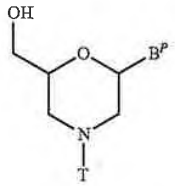
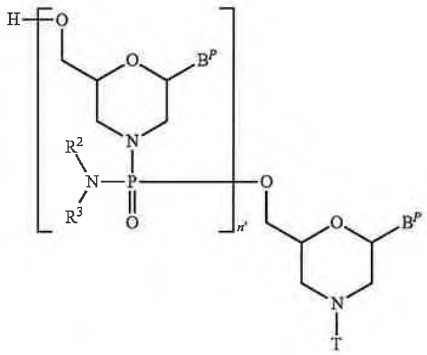
(IIb)



wherein B^P and T have the same significance as defined above.

In Compound (II), the compound represented by general formula (IIb2) below wherein n is 2 to 99 and L is hydrogen can be produced by using Compound (IIb) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

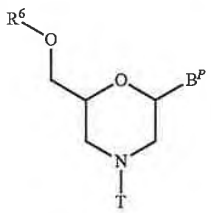
(IIb2)



wherein B^P, n', R^2, R^3 and T have the same significance as defined above.

In Compound (II), the compound represented by general formula (IIc) below wherein n is 1 and L is an acyl can be produced by performing the procedure known as acylation reaction, using Compound (IIb).

(IIc)

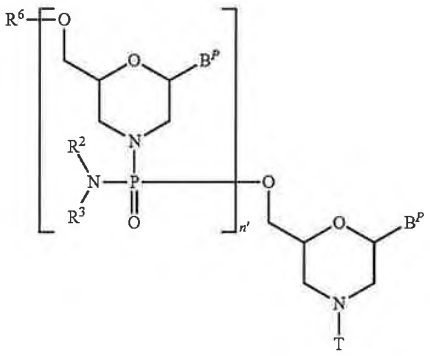


wherein B^P and T have the same significance as defined above; and,

R^5 represents an acyl.

In Compound (II), the compound represented by general formula (IIc2) below wherein n is 2 to 99 and L is an acyl can be produced by using Compound (IIc) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

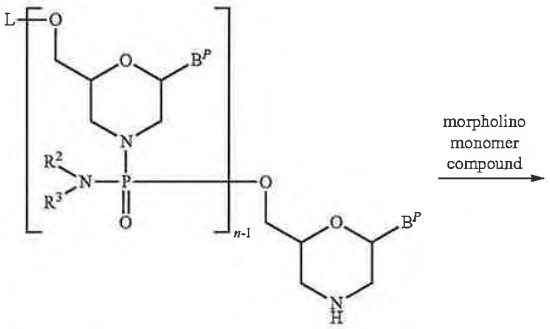
(IIc2)



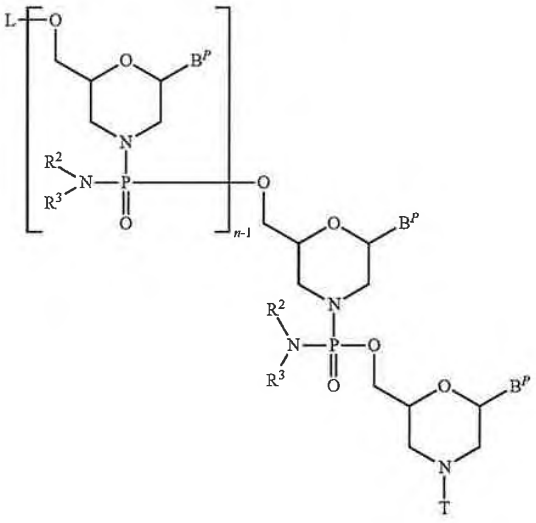
wherein B^P, n', R^2, R^3, R^5 and T have the same significance as defined above.

(2) Step B

Compound (III) is reacted with a morpholino monomer compound in the presence of a base to prepare the compound represented by general formula (VII) below (hereinafter referred to as Compound (VII)):



(III)



(VII)

wherein B^P, L, n, R^2, R^3 and T have the same significance as defined above.

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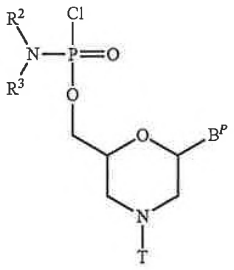
This step can be performed by reacting Compound (III) with the morpholino monomer compound in the presence of a base.

The morpholino monomer compound includes, for example, compounds represented by general formula (VIII) below:

(VIII)



wherein B^P, R^2, R^3 and T have the same significance as defined above.

The "base" which can be used in this step includes, for example, diisopropylamine, triethylamine and N-ethylmorpholine. The amount of the base used is appropriately in a range of 1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (III), preferably, 10 mol equivalents to 100 mol equivalents based on 1 mol of Compound (III).

The morpholino monomer compound and base which can be used in this step may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, N,N-dimethylimidazolidone, N-methylpiperidone, DMF, dichloromethane, acetonitrile, tetrahydrofuran, or a mixture thereof.

The reaction temperature is preferably in a range of, e.g., 0° C. to 100° C., and more preferably, in a range of 10° C. to 50° C.

The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 1 minute to 48 hours in general, and preferably in a range of 30 minutes to 24 hours.

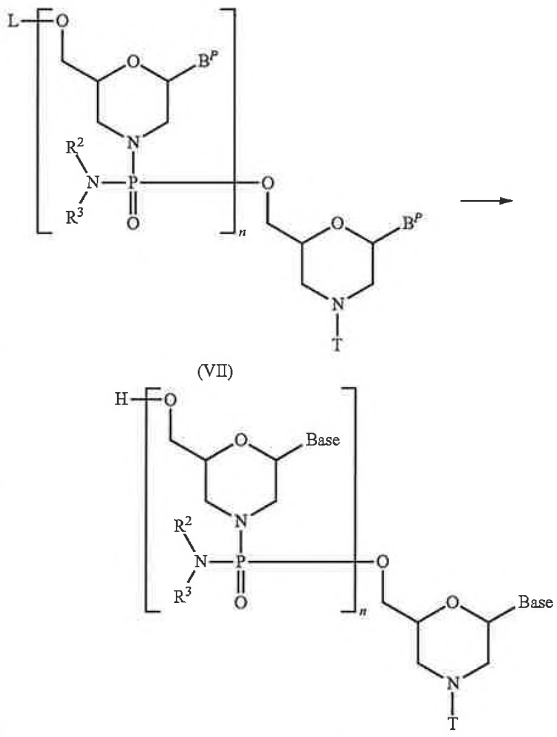
Furthermore, after completion of this step, an acylating agent can be added, if necessary. The "acylating agent" includes, for example, acetic anhydride, acetyl chloride and phenoxyacetic anhydride. The acylating agent may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol(s) (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

If necessary, a base such as pyridine, lutidine, collidine, triethylamine, diisopropylethylamine, N-ethylmorpholine, etc. may also be used in combination with the acylating agent. The amount of the acylating agent is appropriately in a range of 0.1 mol equivalent to 10000 mol equivalents, and preferably in a range of 1 mol equivalent to 1000 mol equivalents. The amount of the base is appropriately in a range of, e.g., 0.1 mol equivalent to 100 mol equivalents, and preferably in a range of 1 mol equivalent to 10 mol equivalents, based on 1 mol of the acylating agent.

The reaction temperature in this reaction is preferably in a range of 10° C. to 50° C., more preferably, in a range of 10° C. to 50° C., much more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C. The reaction time may vary depending upon kind of the acylating agent used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

(3) Step C:

In Compound (VII) produced in Step B, the protective group is removed using a deprotecting agent to prepare the compound represented by general formula (IX).



(VII)

(IX)

wherein Base, B^P, n, R^2, R^3 and T have the same significance as defined above.

This step can be performed by reacting Compound (VII) with a deprotecting agent.

The "deprotecting agent" includes, e.g., conc. ammonia water and methylamine. The "deprotecting agent" used in this step may also be used as a dilution with, e.g., water, methanol, ethanol, isopropyl alcohol, acetonitrile, tetrahydrofuran, DMF, N,N-ditnethylimidazolidone, N-methylpiperidone, or a mixture of these solvents. Among others, ethanol is preferred. The amount of the deprotecting agent used is appropriately in a range of, e.g., 1 mol equivalent to 100000 mol equivalents, and preferably in a range of 10 mol equivalents to 1000 mol equivalents, based on 1 mol of Compound (VII).

The reaction temperature is appropriately in a range of 15° C. to 75° C., preferably, in a range of 40° C. to 70° C., and more preferably, in a range of 50° C. to 60° C. The reaction time for deprotection may vary depending upon kind of Compound (VII), reaction temperature, etc., and is appropriately in a range of 10 minutes to 30 hours, preferably 30 minutes to 24 hours, and more preferably in a range of 5 hours to 20 hours.

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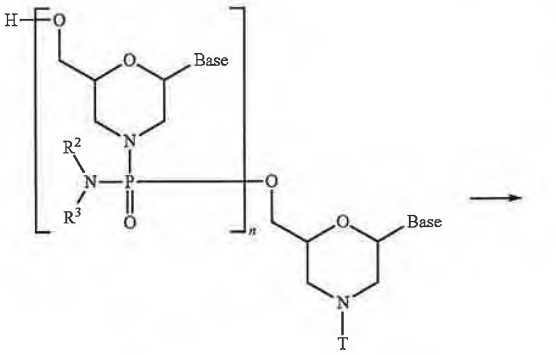
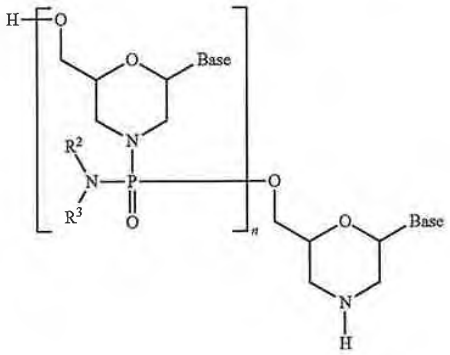
(4) Step D:

PMO (I) is produced by reacting Compound (IX) produced in step C with an acid:



(IX)



(I)

wherein Base, n, R^2, R^3 and T have the same significance as defined above. This step can be performed by adding an acid to Compound (IX).

The "acid" which can be used in this step includes, for example, trichloroacetic acid, dichloroacetic acid, acetic acid, phosphoric acid, hydrochloric acid, etc. The acid used is appropriately used to allow the solution to have a pH range of 0.1 to 4.0, and more preferably, in a range of pH 1.0 to 3.0. The solvent is not particularly limited so long as it is inert to the reaction, and includes, for example, acetonitrile, water, or a mixture of these solvents thereof.

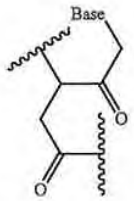
The reaction temperature is appropriately in a range of 10° C. to 50° C., preferably, in a range of 20° C. to 40° C., and more preferably, in a range of 25° C. to 35° C. The reaction time for deprotection may vary depending upon kind of Compound (IX), reaction temperature, etc., and is appropriately in a range of 0.1 minute to 5 hours, preferably 1 minute to 1 hour, and more preferably in a range of 1 minute to 30 minutes.

PMO (I) can be obtained by subjecting the reaction mixture obtained in this step to conventional means of separation and purification such as extraction, concentration, neutralization, filtration, centrifugal separation, recrystallization, reversed phase column chromatography C_8 to C_{18}, cation exchange column chromatography, anion exchange column chromatography, gel filtration column chromatography, high performance liquid chromatography, dialysis, ultrafiltration, etc., alone or in combination thereof. Thus, the desired PMO (I) can be isolated and purified (cf., e.g., WO 1991/09033).

In purification of PMO (I) using reversed phase chromatography, e.g., a solution mixture of 20 mM triethylamine/acetate buffer and acetonitrile can be used as an elution solvent.

In purification of PMO (I) using ion exchange chromatography, e.g., a solution mixture of 1 M saline solution and 10 mM sodium hydroxide aqueous solution can be used as an elution solvent.

A peptide nucleic acid is the oligomer of the present invention having a group represented by the following general formula as the constituent unit:

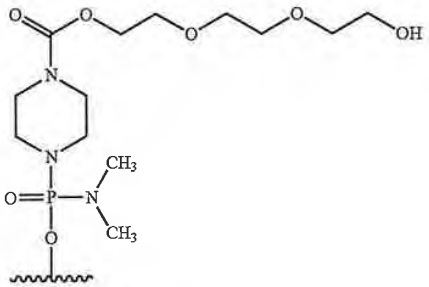


wherein Base has the same significance as defined above.

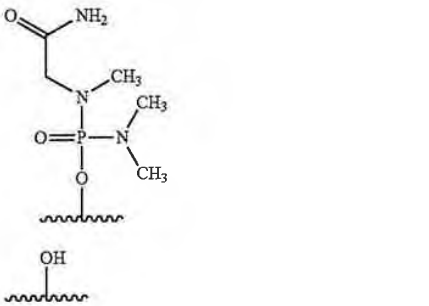
Peptide nucleic acids can be prepared by referring to, e.g., the following literatures.

1) P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, Science, 254, 1497 (1991)
2) M. Egholm, O. Buchardt, P. E. Nielsen, R. H. Berg, Jacs., 114, 1895 (1992)
3) K. L. Dueholmn, M. Egholm, C. Behrens, L. Christensen, H. F. Hansen, T. Vulpius, K. H. Petersen, R. H. Berg, P. E. Nielsen, O. Buchardt, J. Org. Chem., 59, 5767 (1994)
4) L. Christensen, R. Fitzpatrick, B. Gildea, K. H. Petersen, H. F. Hansen, T. Koch, M. Egholm, O. Buchardt, P. E. Nielsen, J. Coull, R. H. Berg, J. Pept. Sci., 1, 175 (1995)
5) T. Koch, H. F. Hansen, P. Andersen, T. Larsen, H. G. Batz, K. Otteson, H. Orum, J. Pept. Res., 49, 80 (1997)

In the oligomer of the present invention, the 5' end may be any of chemical structures (1) to (3) below, and preferably is (3)-OH.



(1)



(2)

(3)

Hereinafter, the groups shown by (1), (2) and (3) above are referred to as "Group (1)," "Group (2)" and "Group (3)," respectively.

2. Pharmaceutical Composition

The oligomer of the present invention causes exon 53 skipping with a higher efficiency as compared to the prior art

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antisense oligomers. It is thus expected that conditions of muscular dystrophy can be relieved with high efficience by administering the pharmaceutical composition comprising the oligomer of the present invention to DMD patients. For example, when the pharmaceutical composition comprising the oligomer of the present invention is used, the same therapeutic effects can be achieved even in a smaller dose than that of the oligomers of the prior art. Accordingly, side effects can be alleviated and such is economical.

In another embodiment, the present invention provides the pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the oligomer of the present invention, a pharmaceutically acceptable salt or hydrate thereof (hereinafter referred to as "the composition of the present invention").

Examples of the pharmaceutically acceptable salt of the oligomer of the present invention contained in the composition of the present invention are alkali metal salts such as salts of sodium, potassium and lithium; alkaline earth metal salts such as salts of calcium and magnesium; metal salts such as salts of aluminum, iron, zinc, copper, nickel, cobalt, etc.; ammonium salts; organic amine salts such as salts of t-octylamine, dibenzylamine, morpholine, glucosamine, phenylglycine alkyl ester, ethylenediamine, N-methylglucamine, guanidine, diethylamine, triethylamine, dicyclohexylamine, N,N-dibenzylethylenediamine, chloroprocaine, procaine, diethanolamine, N-benzylphenethylamine, piperazine, tetramethylammonium, tris(hydroxymethyl)aminomethane; hydrohalide salts such as salts of hydrofluorates, hydrochlorides, hydrobromides and hydroiodides; inorganic acid salts such as nitrates, perchlorates, sulfates, phosphates, etc.; lower alkane sulfonates such as methanesulfonates, trifluoromethanesulfonates and ethanesulfonates; arylsulfonates such as benzenesulfonates and p-toluenesulfonates; organic acid salts such as acetates, malates, fumarates, succinates, citrates, tartarates, oxalates, maleates, etc.; and, amino acid salts such as salts of glycine, lysine, arginine, ornithine, glutamic acid and aspartic acid. These salts may be produced by known methods. Alternatively, the oligomer of the present invention contained in the composition of the present invention may be in the form of a hydrate thereof.

Administration route for the composition of the present invention is not particularly limited so long as it is pharmaceutically acceptable route for administration, and can be chosen depending upon method of treatment. In view of easiness in delivery to muscle tissues, preferred are intravenous administration, intraarterial administration, intramuscular administration, subcutaneous administration, oral administration, tissue administration, transdermal administration, etc. Also, dosage forms which are available for the composition of the present invention are not particularly limited, and include, for example, various injections, oral agents, drips, inhalations, ointments, lotions, etc.

In administration of the oligomer of the present invention to patients with muscular dystrophy, the composition of the present invention preferably contains a carrier to promote delivery of the oligomer to muscle tissues. Such a carrier is not particularly limited as far as it is pharmaceutically acceptable, and examples include cationic carriers such as cationic liposomes, cationic polymers, etc., or carriers using viral envelope. The cationic liposomes are, for example, liposomes composed of 2-O-(2-diethylaminoethyl)carabamoyl-1,3-O-dioleoylglycerol and phospholipids as the essential constituents (hereinafter referred to as "liposome A"), Oligofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectin (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine 2000 (registered trademark) (manufactured by Invitrogen Corp.), DMRIE-C (registered trademark) (manufactured by Invitrogen Corp.), GeneSilencer (registered trademark) (manufactured by Gene Therapy Systems), TransMessenger (registered trademark) (manufactured by QIAGEN, Inc.), TransIT TKO (registered trademark) (manufactured by Mirus) and Nucleofector II (Lonza). Among others, liposome A is preferred. Examples of cationic polymers are JetSI (registered trademark) (manufactured by Qbiogene, Inc.) and Jet-PEI (registered trademark) (polyethylenimine, manufactured by Qbiogene, Inc.). An example of carriers using viral envelop is GenomeOne (registered trademark) (HVJ-E liposome, manufactured by Ishihara Sangyo). Alternatively, the medical devices described in Japanese Patent No. 2924179 and the cationic carriers described in Japanese Domestic Re-Publication PCT Nos. 2006/129594 and 2008/096690 may be used as well.

A concentration of the oligomer of the present invention contained in the composition of the present invention may vary depending on kind of the carrier, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the oligomer of the present invention contained in the composition of the present invention and the carrier (carrier/oligomer of the present invention) may vary depending on property of the oligomer, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20.

In addition to the oligomer of the present invention and the carrier described above, pharmaceutically acceptable additives may also be optionally formulated in the composition of the present invention. Examples of such additives are emulsification aids (e.g., fatty acids having 6 to 22 carbon atoms and their pharmaceutically acceptable salts, albumin and dextran), stabilizers (e.g., cholesterol and phosphatidic acid), isotonizing agents (e.g., sodium chloride, glucose, maltose, lactose, sucrose, trehalose), and pH controlling agents (e.g., hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid, sodium hydroxide, potassium hydroxide and triethanolamine). One or more of these additives can be used. The content of the additive in the composition of the present invention is appropriately 90 wt % or less, preferably 70 wt % or less and more preferably, 50 wt % or less.

The composition of the present invention can be prepared by adding the oligomer of the present invention to a carrier dispersion and adequately stirring the mixture. Additives may be added at an appropriate step either before or after addition of the oligomer of the present invention. An aqueous solvent that can be used in adding the oligomer of the present invention is not particularly limited as far as it is pharmaceutically acceptable, and examples are injectable water or injectable distilled water, electrolyte fluid such as physiological saline, etc., and sugar fluid such as glucose fluid, maltose fluid, etc. A person skilled in the art can appropriately choose conditions for pH and temperature for such matter.

The composition of the present invention may be prepared into, e.g., a liquid form and its lyophilized preparation. The lyophilized preparation can be prepared by lyophilizing the composition of the present invention in a liquid form in a conventional manner. The lyophilization can be performed, for example, by appropriately sterilizing the composition of the present invention in a liquid form, dispensing an aliquot into a vial container, performing preliminary freezing for 2 hours at conditions of about −40 to −20° C., performing a primary drying at 0 to 10° C. under reduced pressure, and then performing a secondary drying at about 15 to 25° C. under reduced pressure. In general, the lyophilized prepa-

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ration of the composition of the present invention can be obtained by replacing the content of the vial with nitrogen gas and capping.

The lyophilized preparation of the composition of the present invention can be used in general upon reconstitution by adding an optional suitable solution (reconstitution liquid) and redissolving the preparation. Such a reconstitution liquid includes injectable water, physiological saline and other infusion fluids. A volume of the reconstitution liquid may vary depending on the intended use, etc., is not particularly limited, and is suitably 0.5 to 2-fold greater than the volume prior to lyophilization or no more than 500 mL.

It is desired to control a dose of the composition of the present invention to be administered, by taking the following factors into account: the type and dosage form of the oligomer of the present invention contained; patients' conditions including age, body weight, etc.; administration route; and the characteristics and extent of the disease. A daily dose calculated as the amount of the oligomer of the present invention is generally in a range of 0.1 mg to 10 g/human, and preferably 1 mg to 1 g/human. This numerical range may vary occasionally depending on type of the target disease, administration route and target molecule. Therefore, a dose lower than the range may be sufficient in some occasion and conversely, a dose higher than the range may be required occasionally. The composition can be administered from once to several times daily or at intervals from one day to several days.

In still another embodiment of the composition of the present invention, there is provided a pharmaceutical composition comprising a vector capable of expressing the oligonucleotide of the present invention and the carrier described above. Such an expression vector may be a vector capable of expressing a plurality of the oligonucleotides of the present invention. The composition may be formulated with pharmaceutically acceptable additives as in the case with the composition of the present invention containing the oligomer of the present invention. A concentration of the expression vector contained in the composition may vary depending upon type of the career, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the expression vector contained in the composition and the carrier (carrier/expression vector) may vary depending on property of the expression vector, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20. The content of the carrier contained in the composition is the same as in the case with the composition of the present invention containing the oligomer of the present invention, and a method for producing the same is also the same as in the case with the composition of the present invention.

Hereinafter, the present invention will be described in more detail with reference to EXAMPLES and TEST EXAMPLES below, but is not deemed to be limited thereto.

EXAMPLES

[Reference Example 1] 4-{[(2S,6R)-(4-amido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl] methoxy}-4-oxobutanoic acid laded onto aminomethyl polystyrene resin

Step 1: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl] meth oxy}-4-oxobutanoic acid

Under argon atmosphere, 22.0 g of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide and 7.04 g of 4-dimethylaminopyridine (4-DMAP) were suspended in 269 mL of dichloromethane, and 5.76 g of succinic anhydride was added to the suspension, followed by stirring at room temperature for 3 hours. To the reaction solution was added 40 mL of methanol, and the mixture was concentrated under reduced pressure. The residue was extracted using ethyl acetate and 0.5M aqueous potassium dihydrogenphosphate solution. The resulting organic layer was washed sequentially with 0.5M aqueous potassium dihydrogenphosphate solution, water and brine in the order mentioned. The resulting organic layer was dried over sodium sulfate and concentrated under reduced pressure to give 25.9 g of the product.

Step 2: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl] methoxy}-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

After 23.5 g of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid was dissolved in 336 mL of pyridine (dehydrated), 4.28 g of 4-DMAP and 40.3 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were added to the solution. Then, 25.0 g of Aminomethyl Polystyrene Resin cross-linked with 1% DVB (manufactured by Tokyo Chemical Industry Co., Ltd., A1543) and 24 mL of triethylamine were added to the mixture, followed by shaking at room temperature for 4 days. After completion of the reaction, the resin was taken out by filtration. The resulting resin was washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure. To the resulting resin were added 150 mL of tetrahydrofuran (dehydrate), 15 mL of acetic anhydride and 15 mL of 2,6-lutidine, and the mixture was shaken at room temperature for 2 hours. The resin was taken out by filtration, washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure to give 33.7 g of the product.

The loading amount of the product was determined by measuring UV absorbance at 409 nm of the molar amount of the trityl per g resin using a known method. The loading amount of the resin was 397.4 μmol/g.

Conditions of UV Measurement
Device: U-2910 (Hitachi, Ltd.)
Solvent: methanesulfonic acid
Wavelength: 265 nm
ϵ Value: 45000

[Reference Example 2] 4-Oxo-4-{[2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}butanoic acid loaded onto 2-aminomethylpolystyrene resin

Step 1: Production of N^2-(phenoxyacetyl)guanosine

Guanosine, 100 g, was dried at 80° C. under reduced pressure for 24 hours. After 500 mL of pyridine (anhydrous) and 500 mL of dichloromethane (anhydrous) were added thereto, 401 mL of chlorotrimethylsilane was dropwise added to the mixture under an argon atmosphere at 0° C., followed by stirring at room temperature for 3 hours. The mixture was again ice-cooled and 66.3 g of phenoxyacetyl chloride was dropwise added thereto. Under ice cooling, the mixture was stirred for further 3 hours. To the reaction solution was added 500 mL of methanol, and the mixture

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was stirred at room temperature overnight. The solvent was then removed by distillation under reduced pressure. To the residue was added 500 mL of methanol, and concentration under reduced pressure was performed 3 times. To the residue was added 4 L of water, and the mixture was stirred for an hour under ice cooling. The precipitates formed were taken out by filtration, washed sequentially with water and cold methanol and then dried to give 150.2 g of the objective compound (yield: 102%) (cf.: Org. Lett. (2004), Vol. 6, No. 15, 2555-2557).

Step 2: N-{9-[(2R,6S)-6-(hydroxymethyl)-4-morpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide p-toluenesulfonate

In 480 mL of methanol was suspended 30 g of the compound obtained in Step 1, and 130 mL, of 2N hydrochloric acid was added to the suspension under ice cooling. Subsequently, 56.8 g of ammonium tetraborate tetrahydrate and 16.2 g of sodium periodate were added to the mixture in the order mentioned and stirred at room temperature for 3 hours. The reaction solution was ice cooled and the insoluble matters were removed by filtration, followed by washing with 100 mL of methanol. The filtrate and washing liquid were combined and the mixture was ice cooled. To the mixture was added 11.52 g of 2-picoline borane. After stirring for 20 minutes, 54.6 g of p-toluenesulfonic acid monohydrate was slowly added to the mixture, followed by stirring at 4° C. overnight. The precipitates were taken out by filtration and washed with 500 mL of cold methanol and dried to give 17.7 g of the objective compound (yield: 43.3%).

^{1}H NMR (8, DMSO-d6): 9.9-9.2 (2H, br), 8.35 (1H, s), 7.55 (2H, m), 7.35 (2H, m), 7.10 (2H, d, J=7.82 Hz), 7.00 (3H, m), 5.95 (1H, dd, J=10.64, 2.42 Hz), 4.85 (2H, s), 4.00 (1H, m), 3.90-3.60 (2H, m), 3.50-3.20 (5H, m), 2.90 (1H, m), 2.25 (3H, s)

Step 3: Production of N-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide

In 30 mL of dichloromethane was suspended 2.0 g of the compound obtained in Step 2, and 13.9 g of triethylamine and 18.3 g of trityl chloride were added to the suspension under ice cooling. The mixture was stirred at room temperature for an hour. The reaction solution was washed with saturated sodium bicarbonate aqueous solution and then with water, and dried. The organic layer was concentrated under reduced pressure. To the residue was added 40 mL of 0.2M sodium citrate buffer (pH 3)/methanol (1:4 (v/v)), and the mixture was stirred. Subsequently, 40 mL of water was added and the mixture was stirred for an hour under ice cooling. The mixture was taken out by filtration, washed with cold methanol and dried to give 1.84 g of the objective compound (yield: 82.0%).

Step 4: Production of 4-oxo-4-{[(2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}butanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that N-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

[Reference Example 3] 4-{[(2S,6R)-6-(5-Methyl-2,4-dioxo-3,4-dihydropyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-5-methylpyrimidine-2,4(1H,3H)-dione was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

[Reference Example 4] 1,12-Dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 2-[2-(2-hydroxyethoxyl)ethoxy]ethyl 4-tritylpiperazine-1-carboxylic acid (the compound described in WO 2009/064471) was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide.

According to the descriptions in EXAMPLES 1 to 12 and REFERENCE EXAMPLES 1 to 3 below, various types of PMO shown by PMO Nos. 1-11 and 13-16 in TABLE 2 were synthesized. The PMO synthesized was dissolved in injectable water (manufactured by Otsuka Pharmaceutical Factory, Inc.). PMO No. 12 was purchased from Gene Tools, LLC.

TABLE 2

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
1	31-55	5' end: group (3)	SEQ ID NO: 4
2	32-53	5' end: group (3)	SEQ ID NO: 8
3	32-56	5' end: group (3)	SEQ ID NO: 11
4	33-54	5' end: group (3)	SEQ ID NO: 15
5	34-58	5' end: group (3)	SEQ ID NO: 25
6	36-53	5' end: group (3)	SEQ ID NO: 32
7	36-55	5' end: group (3)	SEQ ID NO: 34
8	36-56	5' end: group (3)	SEQ ID NO: 35
9	36-57	5' end: group (3)	SEQ ID NO: 36

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TABLE 2-continued

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
10	33-57	5' end: group (3)	SEQ ID NO: 18
11	39-69	Sequence corresponding to H53A(+39 + 69) (cf. Table 1) in Non-Patent Document 3, 5' end: group (3)	SEQ ID NO: 38
12	30-59	Sequence corresponding to h53A30/1 (cf. Table 1) in Non-Patent Document 5, 5' end: group (2)	SEQ ID NO: 39
13	32-56	5' end: group (1)	SEQ ID NO: 11
14	36-56	5' end: group (1)	SEQ ID NO: 35
15	30-59	Sequence corresponding to h53A30/1 cf. Table 1) in Non-Patent Document 5 5' end: group (3)	SEQ ID NO: 39
16	23-47	Sequence corresponding to SEQ ID NO: 429 described in Patent Document 4, 5' end: group (3)	SEQ ID NO: 47

Example 1

PMO No. 8

4-{[(2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid, loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 1), 2 g (800 μmol) was transferred to a reaction vessel, and 30 mL of dichloromethane was added thereto. The mixture was allowed to stand for 30 minutes. After the mixture was further washed twice with 30 mL of dichloromethane, the following synthesis cycle was started. The desired morpholino monomer compound was added in each cycle to give the nucleotide sequence of the title compound.

TABLE 3

Step	Reagent	Volume (mL)	Time (min)
1	deblocking solution	30	2.0
2	deblocking solution	30	2.0
3	deblocking solution	30	2.0
4	deblocking solution	30	2.0
5	deblocking solution	30	2.0
6	deblocking solution	30	2.0
7	neutralizing solution	30	1.5
8	neutralizing solution	30	1.5
9	neutralizing solution	30	1.5
10	neutralizing solution	30	1.5
11	neutralizing solution	30	1.5
12	neutralizing solution	30	1.5
13	dichloromethane	30	0.5
14	dichloromethane	30	0.5
15	dichloromethane	30	0.5
16	coupling solution B	20	0.5
17	coupling solution A	6-11	90.0
18	dichloromethane	30	0.5
19	dichloromethane	30	0.5
20	dichloromethane	30	0.5
21	capping solution	30	3.0
22	capping solution	30	3.0
23	dichloromethane	30	0.5
24	dichloromothane	30	0.5
25	dichloromethane	30	0.5

The deblocking solution used was a solution obtained by dissolving a mixture of trifluoroacetic acid (2 equivalents) and triethylamine (1 equivalent) in a dichloromethane solution containing 1% (v/v) ethanol and 10% (v/v) 2,2,2-trifluoroethanol to be 3% (w/v). The neutralizing solution used was a solution obtained by dissolving N,N-diisopropylethylamine in a dichloromethane solution containing 25% (v/v) 2-propanol to be 5% (v/v). The coupling solution A used was a solution obtained by dissolving the morpholino monomer compound in 1,3-dimethyl-2-imidazolidinone containing 10% (v/v) N,N-diisopropylethylamine to be 0.15M. The coupling solution B used was a solution obtained by dissolving N,N-diisopropylethylamine in 1,3-dimethyl-2-imidazolidinone to be 10% (v/v). The capping solution used was a solution obtained by dissolving 20% (v/v) acetic anhydride and 30% (v/v) 2,6-lutidine in dichloromethane.

The aminomethyl polystyrene resin loaded with the PMO synthesized above was recovered from the reaction vessel and dried at room temperature for at least 2 hours under reduced pressure. The dried PMO loaded onto aminomethyl polystyrene resin was charged in a reaction vessel, and 200 mL of 28% ammonia water-ethanol (1/4) was added thereto. The mixture was stirred at 55° C. for 15 hours. The aminomethyl polystyrene resin was separated by filtration and washed with 50 mL of water-ethanol (1/4). The resulting filtrate was concentrated under reduced pressure. The resulting residue was dissolved in 100 mL of a solvent mixture of 20 mM acetic acid-triethylamine buffer (TEAA buffer) and acetonitrile (4/1) and filtered through a membrane filter. The filtrate obtained was purified by reversed phase HPLC. The conditions used are as follows.

TABLE 4

Column	XTerra MS18 (Waters, ϕ50 × 100 mm, 1CV = 200 mL)
Flow rate	60 mL rain
Column temperature	room temperature
Solution A	20 mM TEAA buffer
Solution B	CH_3CN
Gradient	(B) conc. 20 → 50%/9CV

Each fraction was analyzed and the product was recovered in 100 mL of acetonitrile-water (1/1), to which 200 mL of ethanol was added. The mixture was concentrated under reduced pressure. Further drying under reduced pressure gave a white solid. To the resulting solid was added 300 mL of 10 mM phosphoric acid aqueous solution to suspend the solid. To the suspension was added 10 mL of 2M phosphoric acid aqueous solution, and the mixture was stirred for 15 minutes. Furthermore, 15 mL of 2M sodium hydrate aqueous solution was added for neutralization. Then, 15 mL of 2M sodium hydroxide aqueous solution was added to make the mixture alkaline, followed by filtration through a membrane filter (0.45 μm). The mixture was thoroughly washed

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with 100 mL of 10 mM sodium hydroxide aqueous solution to give the product as an aqueous solution.

The resulting aqueous solution containing the product was purified by an anionic exchange resin column. The conditions used are as follows.

TABLE 5

Column	Source 30Q (GE Healthcare,ϕ 40 × 150 mm, 1CV = 200 mL)
Flow rate	80 mL/min
Column temp.	room temperature
Solution A	10 mM sodium hydroxide aqueous solution
Solution B	10 mM sodium hydroxide aqueous solution, 1M sodium chloride aqueous solution
Gradient	(B) conc. 5 → 35%/15CV

Each fraction was analyzed (on HPLC) and the product was obtained as an aqueous solution. To the resulting aqueous solution was added 225 mL of 0.1M phosphate buffer (pH 6.0) for neutralization. The mixture was filtered through a membrane filter (0.45 μm). Next, ultrafiltration was performed under the conditions

TABLE 6

Filter	PELLICON2 MINI FILTER PLBC 3K Regenerated Cellulose, Screen Type C
Size	0.1 m^2

The filtrate was concentrated to give approximately 250 mL of an aqueous solution. The resulting aqueous solution was filtered through a membrane filter (0.45 μm). The aqueous solution obtained was freeze-dried to give 1.5 g of the objective compound as a white cotton-like solid.

ESI-TOF-MS Calcd.: 6924.82.
Found: 6923.54.

Example 2

PMO. No. 1

The title compound was produced in accordance with the procedure of EXAMPLE 1.

MALDI-TOF-MS Calcd.: 8291.96.
Found: 8296.24.

Example 3

PMO. No. 2

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 7310.13.
Found: 7309.23.

Example 4

PMO. No. 3

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8270.94.
Found: 8270.55.

Example 5

PMO. No. 4

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-(2H)-yl)-4-trityl-morpholin-2-yl)methoxy)-4-oxobutanoic acid (REFERENCE EXAMPLE 3) loaded onto aminomethyl polystyrene resin was used as the starting material.

ESI-TOF-MS Calcd.: 7310.13.
Found: 7310.17.

Example 6

PMO. No. 5

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-trityl-morpholin-2-yl)methoxy)-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 3) was used as the starting material.

ESI-TOF-MS Calcd.: 8270.94.
Found: 8270.20.

Example 7

PMO. No. 6

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 5964.01.
Found: 5963.68.

Example 8

PMO. No. 7

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 6609.55.
Found: 6608.85.

Example 9

PMO. No. 9

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9(6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 7280.11.
Found: 7279.4.

Example 10

PMO. No. 10

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-

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6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9(6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 8295.95.

Found: 8295.91.

Example 11

PMO. No. 13

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 7276.15.

Found: 7276.69.

Example 12

PMO. No. 14

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-5-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 8622.27.

Found: 8622.29.

Comparative Example 1

PMO. No. 11

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 10274.63.

Found: 10273.71.

Comparative Example 2

PMO. No. 15

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 9941.33.

Found: 9940.77.

Comparative Example 3

PMO. No. 16

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8238.94.

Found: 8238.69.

Test Example 1

In Vitro Assay

Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 10 μM of the oligomers PMO Nos. 1 to 8 of the present invention and the antisense oligomer PMO No. 11 were transfected with 4×10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

After transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen) under conditions of 37° C. and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

94° C., 2 mins: thermal denaturation

[94° C., 10 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer: (SEQ ID NO: 40)
5'-AGGATTTGGAACAGAGGCGTC-3'

Reverse primer: (SEQ ID NO: 41)
5'-GTCTGCCACTGGCGGAGGTC-3'

Next, a nested PCR was performed with the product amplified by RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94° C., 2 mins: thermal denaturation

[94° C., 15 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

Forward primer: (SEQ ID NO: 42)
5'-CATCAAGCAGAAGGCAACAA-3'

Reverse primer: (SEQ ID NO: 43)
5'-GAAGTTTCAGGGCCAAGTCA-3'

The reaction product, 1 μl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A+B)\times 100$$

Experimental Results

The results are shown in FIG. 1. This experiment revealed that the oligomers PMO Nos. 1 to 8 of the present invention

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caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomer PMO No. 11. In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than four times higher exon skipping efficiency than that of the antisense oligomer PMO No. 11.

Test Example 2

In Vitro Assay Using Human Fibroblasts

Human myoD gene (SEQ ID NO: 44) was introduced into TIG-119 cells (human normal tissue-derived fibroblasts, National Institute of Biomedical Innovation) or 5017 cells (human DMD patient-derived fibroblasts, Coriell Institute for Medical Research) using a ZsGreen1 coexpression retroviral vector.

After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at $5\times10^4/cm^2$ into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM.F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

The medium was replaced 24 hours later by differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12 to 14 days to differentiate into myotubes.

Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and the morpholino oligomer was added thereto in a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 1 mins; 60° C., 1 mins; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 7 mins: final extension

The primers used were hEX51F and hEX55R.

hEX51F: (SEQ ID NO: 45)
5'-CGGGCTTGGACAGAACTTAC-3'

hEx55R: (SEQ ID NO: 46)
5'-TCCTTACGGGTAGCATCCTG-3'

The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

Skipping efficiency (%)=$A/(A+B)\times100$

Experimental Results

The results are shown in FIGS. **2** and **3**. This experiment revealed that in TIG-119 cells, the oligomers PMO Nos. 3, 8 and 9 of the present invention (FIG. **2**) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. **2**). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than twice higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. **2**).

Furthermore, this experiment revealed that the oligomers PMO Nos. 3 and 8 to 10 of the present invention (FIG. **3**) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. **3**). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than seven times higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. **3**).

Test Example 3

In Vitro Assay Using Human Fibroblasts

The skin fibroblast cell line (fibroblasts from human DMD patient (exons 45-52 or exons 48-52)) was established by biopsy from the medial left upper arm of DMD patient with deletion of exons 45-52 or DMD patient with deletion of exons 48-52. Human myoD gene (SEQ ID NO: 44) was introduced into the fibroblast cells using a ZsGreen1 coexpression retroviral vector.

After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at $5\times10^4/cm^2$ into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

The medium was replaced 24 hours later by a differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12, 14 or 20 days to differentiate into myotubes.

Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and a morpholino oligomer was added thereto at a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 1 mins; 60° C., 1 mins; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 7 mins: final extension

The primers used were hEx44F and h55R.

hEx44F: (SEQ ID NO: 48)
5'-TGTTGAGAAATGGCGGCGT-3'

hEx55R: (SEQ ID NO: 46)
5'-TCCTTACGGGTAGCATCCTG-3'

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The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

$$\text{Skipping efficiency (\%)} = A/(A+B) \times 100$$

Experimental Results

The results are shown in FIGS. **4** and **5**. This experiment revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with an efficiency as high as more than 80% in the cells from DMD patient with deletion of exons 45-52 (FIG. **4**) or deletion of exons 48-52 (FIG. **5**). Also, the oligomers PMO Nos. 3 and 8 of the present invention were found to cause exon 53 skipping with a higher efficiency than that of the antisense oligomer PMO No. 15 in the cells from DMD patient with deletion of exons 45-52 (FIG. **4**).

Test Example 4

Western Blotting

The oligomer PMO No. 8 of the present invention was added to the cells at a concentration of 10 μM, and proteins were extracted from the cells after 72 hours using a RIPA buffer (manufactured by Thermo Fisher Scientific) containing Complete Mini (manufactured by Roche Applied Science) and quantified using a BCA protein assay kit (manufactured by Thermo Fisher Scientific). The proteins were electrophoresed in NuPAGE Novex Tris-Acetate Gel 3-8% (manufactured by Invitrogen) at 150V for 75 minutes and transferred onto a PVDF membrane (manufactured by Millipore) using a semi-dry blotter. The PVDF membrane was blocked with a 5% ECL Blocking agent (manufactured by GE Healthcare) and the membrane was then incubated in a solution of anti-dystrophin antibody (manufactured by NCL-Dys1, Novocastra). After further incubation in a solution of peroxidase-conjugated goat-antimouse IgG (Model No. 170-6516, Bio-Rad), the membrane was stained with ECL Plus Western blotting system (manufactured by GE Healthcare).

Immunostaining

The oligomer PMO No. 3 or 8 of the present invention was added to the cells. The cells after 72 hours were fixed in 3% paraformaldehyde for 10 minutes, followed by incubation in 10% Triton-X for 10 minutes. After blocking in 10% goat serum-containing PBS, the membrane was incubated in a solution of anti-dystrophin antibody (NCL-Dys1, Novocastra). The membrane was further incubated in a solution of anti-mouse IgG antibody (manufactured by Invitrogen). The membrane was mounted with Pro Long Gold Antifade reagent (manufactured by Invitrogen) and observed with a fluorescence microscope.

Experimental Results

The results are shown in FIGS. **6** and **7**. In this experiment it was confirmed by western blotting (FIG. **6**) and immunostaining (FIG. **7**) that the oligomers PMO Nos. 3 and 8 of the present invention induced expression of the dystrophin protein.

Test Example 5

In Vitro Assay Using Human Fibroblasts

The experiment was performed as in TEST EXAMPLE 3.

Experimental Results

The results are shown in FIG. **8**. This experiment revealed that in the cells from DMD patients with deletion of exons 45-52, the oligomers PMO Nos. 3 to 8 of the present invention caused exon 53 skipping with a higher efficiency than the oligomers PMO Nos. 13 and 14 of the present invention (FIG. **8**).

Test Example 6

In Vitro Assay

Experiments were performed using the antisense oligomers of 2'-O-methoxy-phosphorothioates (2'-OMe-S-RNA) shown by SEQ ID NO: 49 to SEQ ID NO: 123. Various antisense oligomers used for the assay were purchased from Japan Bio Services. The sequences of various antisense oligomers are given below.

TABLE 7

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_39-69	CAUUCAACUGUUGCCUCCGGUUCUGAAGGUG	49
H53_1-25	UCCCACUGAUUCUGAAUUCUUUCAA	50
H53_6-30	CUUCAUCCCACUGAUUCUGAAUUCU	51
H53_11-35	UUGUACUUCAUCCCACUGAUUCUGA	52
H53_16-40	UGUUCUUGUACUUCAUCCCACUGAU	53
H53_21-45	GAAGGUGUUCUUGUACUUCAUCCCA	54
H53_26-50	GUUCUGAAGGUGUUCUUGUACUUCA	55
H53_31-55	CUCCGGUUCUGAAGGUGUUCUUGUA	56
H53_36-60	GUUGCCUCCGGUUCUGAAGGUGUUC	57
H53_41-65	CAACUGUUGCCUCCGGUUCUGAAGG	58
H53_46-70	UCAUUCAACUGUUGCCUCCGGUUCU	59
H53_51-75	ACAUUUCAUUCAACUGUUGCCUCCG	60
H53_56-80	CUUUAACAUUUCAUUCAACUGUUGC	61
H53_61-85	GAAUCCUUUAACAUUUCAUUCAACU	62
H53_66-90	GUGUUGAAUCCUUUAACAUUUCAUU	63
H53_71-95	CCAUUGUGUUGAAUCCUUUAACAUU	64
H53_76-100	UCCAGCCAUUGUGUUGAAUCCUUUA	65
H53_81-105	UAGCUUCCAGCCAUUGUGUUGAAUC	66
H53_86-110	UUCCUUAGCUUCCAGCCAUUGUGUU	67
H53_91-115	GCUUCUUCCUUAGCUUCCAGCCAUU	68

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TABLE 7-continued

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_96-120	GCUCAGCUUCUUCCUUAGCUUCCAG	69
H53_101-125	GACCUGCUCAGCUUCUUCCUUAGCU	70
H53_106-130	CCUAAGACCUGCUCAGCUUCUUCCU	71
H53_111-135	CCUGUCCUAAGACCUGCUCAGCUUC	72
H53_116-140	UCUGGCCUGUCCUAAGACCUGCUCA	73
H53_121-145	UUGGCUCUGGCCUGUCCUAAGACCU	74
H53_126-150	CAAGCUUGGCUCUGGCCUGUCCUAA	75
H53_131-155	UGACUCAAGCUUGGCUCUGGCCUGU	76
H53_136-160	UUCCAUGACUCAAGCUUGGCUCUGG	77
H53_141-165	CCUCCUUCCAUGACUCAAGCUUGGC	78
H53_146-170	GGGACCCUCCUUCCAUGACUCAAGC	79
H53_151-175	GUAUAGGGACCCUCCUUCCAUGACU	80
H53_156-180	CUACUGUAUAGGGACCCUCCUUCCA	81
H53_161-185	UGCAUCUACUGUAUAGGGACCCUCC	82
H53_166-190	UGGAUUGCAUCUACUGUAUAGGGAC	83
H53_171-195	UCUUUUGGAUUGCAUCUACUGUAUA	84
H53_176-200	GAUUUUCUUUUGGAUUGCAUCUACU	85
H53_181-205	UCUGUGAUUUUCUUUUGGAUUGCAU	86
H53_186-210	UGGUUUCUGUGAUUUUCUUUUGGAU	87
H53_84-108	CCUUAGCUUCCAGCCAUUGUGUUGA	88
H53_88-112	UCUUCCUUAGUUCCAGCCAUUGUG	89
H53_119-143	GGCUCUGGCCUGUCCUAAGACCUGC	90
H53_124-148	AGCUUGGCUCUGGCCUGUCCUAAGA	91
H53_128-152	CUCAAGCUUGGCUCUGGCCUGUCCU	92
H53_144-168	GACCCUCCUUCCAUGACUCAAGCUU	93
H53_149-173	AUAGGGACCCUCCUUCCAUGACUCA	94
H53_153-177	CUGUAUAGGGACCCUCCUUCCAUCGA	95
H53_179-203	UGUGAUUUUCUUUUGGAUUGCAUCU	96
H53_184-208	GUUUCUGUGAUUUUCUUUUGGAUUG	97
H53_188-212	CUUGGUUUCUGUGAUUUUCUUUUGG	98
H53_29-53	CCGGUUCUGAAGGUGUUCUUGUACU	99
H53_30-54	UCCGGUUCUGAAGGUGUUCUUGUAC	100
H53_32-56	CCUCCGGUUCUGAAGGUGUUCUUGU	101
H53_33-57	GCCUCCGGUUCUGAAGGUGUUCUUG	102
H53_34-58	UGCCUCCGGUUCUGAAGGUGUUCUU	103
H53_35-59	UUGCCUCCGGUUCUGAAGGUGUUCU	104
H53_37-61	UGUUGCCUCCGGUUCUGAAGGUGUU	105
H53_38-62	CUGUUGCCUCCGGUUCUGAAGGUGU	106
H53_39-63	ACUGUUGCCUCCGGUUCUGAAGGUG	107

TABLE 7-continued

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_40-64	AACUGUUGCCUCCGGUUCUGAAGGU	108
H53_32-61	UGUUGCCUCCGGUUCUGAAGGUGUUCUUGU	109
H53_32-51	GGUUCUGAAGGUGUUCUUGU	110
H53_35-54	UCCGGUUCUGAAGGUGUUCU	111
H53_37-56	CCUCCGGUUCUGAAGGUGUU	112
H53_40-59	UUGCCUCCGGUUCUGAAGGU	113
H53_42-61	UGUUGCCUCCGGUUCUGAAG	114
H53_32-49	UUCUGAAGGUGUUCUUGU	115
H53_35-52	CGGUUCUGAAGGUGUUCU	116
H53_38-55	CUCCGGUUCUGAAGGUGU	117
H53_41-58	UGCCUCCGGUUCUGAAGG	118
H53_44-61	UGUUGCCUCCGGUUCUGA	119
H53_35-49	UUCUGAAGGUGUUCU	120
H53_40-54	UCCGGUUCUGAAGGU	121
H53_45-59	UUGCCUCCGGUUCUG	122
H53_45-62	CUGUUGCCUCCGGUUCUG	123

RD cells (human rhabdomyosarcomna cell line) were plated at 3×10^5 in a 6-well plate and cultured in 2 nil, of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37° C. and 5% CO_2 overnight. Complexes of various antisense oligomers (Japan Bio Services) (1 μM) for exon 53 skipping and Lipofectamine 2000 (manufactured by Invitrogen Corp.) were prepared and 200 μl was added to RD cells where 1.8 mL of the medium was exchanged, to reach the final concentration of 100 nM.

After completion of the addition, the cells were cultured overnight. The cells were washed twice with PBS (manufactured by Nissui, hereafter the same) and then 500 μl of ISOGEN (manufactured by Nippon Gene) were added to the cells. After the cells were allowed to stand at room temperature for a few minutes for cell lysis, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

94° C., 2 mins: thermal denaturation

[94° C., 10 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

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Forward primer:
(SEQ ID NO: 42)
5'CATCAAGCAGAAGGCAACAA-3'

Reverse primer:
(SEQ ID NO: 43)
5'-GAAGTTTCAGGGCCAAGTCA-3'

Subsequently, a nested PCR was performed with the amplified product of RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94° C., 2 mins: thermal denaturation

[94° C., 15 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

Forward primer:
(SEQ. ID NO: 40)
5'-AGGATTTGGAACAGAGGCGTC-3'

Reverse primer:
(SEQ ID NO: 41)
5'-GTCTGCCACTGGCGGAGGTC-3'

The reaction product, 1 μl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A+B)\times 100$$

Experimental Results

The results are shown in FIGS. **9** to **17**. These experiments revealed that, when the antisense oligomers were designed at exons 31-61 from the 5' end of exon 53 in the human dystrophin gene, exon 53 skipping could be caused with a high efficiency.

Test Example 7

Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 0.3 to 30 μM of the antisense oligomers were transfected with 3.5×10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

After the transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37° C. and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was then added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit (manufactured by Qiagen, Inc.). A reaction solution was prepared in accordance with the protocol attached to the kit. The thermal cycler used was a PTC-100 (manufactured by MJ Research). The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 30 seconds; 60° C., 30 seconds; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 10 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer:
(SEQ. ID NO: 42)
5'-CATCAAGCAGAAGGCAACAA-3'

Reverse primer:
(SEQ. ID NO: 43)
5'-GAAGTTTCAGGCCAAGTCA-3'

The reaction product, 1 μl, of the PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A+B)\times 100$$

Experimental Results

The results are shown in FIGS. **18** and **19**. These experiments revealed that the oligomer PMO No. 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomers PMO Nos. 15 and 16 (FIG. **18**). It was also revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the oligomers PMO Nos. 13 and 14 of the present invention (FIG. **19**). These results showed that the sequences with —OH group at the 5' end provide a higher skipping efficiency even in the same sequences.

INDUSTRIAL APPLICABILITY

Experimental results in TEST EXAMPLES demonstrate that the oligomers of the present invention (PMO Nos. 1 to 10) all caused exon 53 skipping with a markedly high efficiency under all cell environments, as compared to the oligomers (PMO Nos. 11, 12, 15 and 16) in accordance with the prior art. The 5017 cells used in TEST EXAMPLE 2 are the cells isolated from DMD patients, and the fibroblasts used in TEST EXAMPLES 3 and 5 are exon 53 skipping target cells from DMD patients. Particularly in TEST EXAMPLES 3 and 5, the oligomers of the present invention show the exon 53 skipping efficiency of 90% or higher in the cells from DMD patients that are the target for exon 53 skipping. Consequently, the oligomers of the present invention can induce exon 53 skipping with a high efficiency, when DMD patients are administered.

Therefore, the oligomers of the present invention are extremely useful for the treatment of DMD.

Sequence Listing Free Text

SEQ ID NO: 2: synthetic nucleic acid
SEQ ID NO: 3: synthetic nucleic acid
SEQ ID NO: 4: synthetic nucleic acid
SEQ ID NO: 5: synthetic nucleic acid
SEQ ID NO: 6: synthetic nucleic acid
SEQ ID NO: 7: synthetic nucleic acid
SEQ ID NO: 8: synthetic nucleic acid

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SEQ ID NO: 9: synthetic nucleic acid
SEQ ID NO: 10: synthetic nucleic acid
SEQ ID NO: 11: synthetic nucleic acid
SEQ ID NO: 12: synthetic nucleic acid
SEQ ID NO: 13: synthetic nucleic acid
SEQ ID NO: 14: synthetic nucleic acid
SEQ ID NO: 15: synthetic nucleic acid
SEQ ID NO: 16: synthetic nucleic acid
SEQ ID NO: 17: synthetic nucleic acid
SEQ ID NO: 18: synthetic nucleic acid
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SEQ ID NO: 103: synthetic nucleic acid
SEQ ID NO: 104: synthetic nucleic acid
SEQ ID NO: 105: synthetic nucleic acid
SEQ ID NO: 106: synthetic nucleic acid
SEQ ID NO: 107: synthetic nucleic acid
SEQ ID NO: 108: synthetic nucleic acid
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SEQ ID NO: 111: synthetic nucleic acid
SEQ ID NO: 112: synthetic nucleic acid
SEQ ID NO: 113: synthetic nucleic acid
SEQ ID NO: 114: synthetic nucleic acid
SEQ ID NO: 115: synthetic nucleic acid
SEQ ID NO: 116: synthetic nucleic acid
SEQ ID NO: 117: synthetic nucleic acid
SEQ ID NO: 118: synthetic nucleic acid
SEQ ID NO: 119: synthetic nucleic acid
SEQ ID NO: 120: synthetic nucleic acid
SEQ ID NO: 121: synthetic nucleic acid
SEQ ID NO: 122: synthetic nucleic acid
SEQ ID NO: 123: synthetic nucleic acid

Sequence Listing

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 123

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<213> ORGANISM: Homo sapiens

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aggtcttagg acaggccaga gccaagcttg agtcatggaa ggagggtccc tatacagtag 180

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tccggttctg aaggtgttc 19

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<400> SEQUENCE: 34

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cctccggttc tgaaggtgtt c 21

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<400> SEQUENCE: 36

gcctccggtt ctgaaggtgt tc 22

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tgcctccggt tctgaaggtg ttc 23

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<211> LENGTH: 31
<212> TYPE: DNA
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<400> SEQUENCE: 39

ttgcctccgg ttctgaaggt gttcttgtac 30

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aggatttgga acagaggcgt c 21

<210> SEQ ID NO 41
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<212> TYPE: DNA
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gtctgccact ggcggaggtc 20

<210> SEQ ID NO 42
<211> LENGTH: 20
<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 42

catcaagcag aaggcaacaa 20

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<400> SEQUENCE: 43

gaagtttcag ggccaagtca 20

<210> SEQ ID NO 44
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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 44

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cgcttcttcg aagacctgga cccgcgcctg atgcacgtgg gcgcgctcct gaaacccgaa 180

gagcactcgc acttccccgc ggcggtgcac ccggccccgg gcgcacgtga ggacgagcat 240

gtgcgcgcgc ccagcgggca ccaccaggcg ggccgctgcc tactgtgggc ctgcaaggcg 300

tgcaagcgca agaccaccaa cgccgaccgc cgcaaggccg ccaccatgcg cgagcggcgc 360

cgcctgagca aagtaaatga ggcctttgag acactcaagc gctgcacgtc gagcaatcca 420

aaccagcggt tgcccaaggt ggagatcctg cgcaacgcca tccgctatat cgagggcctg 480

caggctctgc tgcgcgacca ggacgccgcg ccccctggcg ccgcagccgc cttctatgcg 540

ccgggcccgc tgcccccggg ccgcggcggc gagcactaca gcggcgactc cgacgcgtcc 600

agcccgcgct ccaactgctc cgacggcatg atggactaca gcggcccccc gagcggcgcc 660

cggcggcgga actgctacga aggcgcctac tacaacgagg cgcccagcga acccaggccc 720

gggaagagtg cggcggtgtc gagcctagac tgcctgtcca gcatcgtgga gcgcatctcc 780

accgagagcc ctgcggcgcc cgccctcctg ctggcggacg tgccttctga gtcgcctccg 840

cgcaggcaag aggctgccgc ccccagcgag ggagagagca gcggcgaccc cacccagtca 900

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tga 963

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<400> SEQUENCE: 45

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<210> SEQ ID NO 46
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 46

tccttacggg tagcatcctg 20

<210> SEQ ID NO 47
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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 47

ctgaaggtgt tcttgtactt catcc 25

<210> SEQ ID NO 48
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial

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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 48

tgttgagaaa tggcggcgt 19

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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 49

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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

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ucccacugau ucugaauucu uucaa 25

<210> SEQ ID NO 51
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<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 51

cuucauccca cugauucuga auucu 25

<210> SEQ ID NO 52
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<400> SEQUENCE: 52

uuguacuuca ucccacugau ucuga 25

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<400> SEQUENCE: 53

uguucuugua cuucauccca cugau 25

<210> SEQ ID NO 54
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 54

gaagguguuc uuguacuuca uccca 25

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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 55

guucugaagg uguucuugua cuuca 25

<210> SEQ ID NO 56
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<212> TYPE: RNA
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<400> SEQUENCE: 56

cuccgguucu gaagguguuc uugua 25

<210> SEQ ID NO 57
<211> LENGTH: 25
<212> TYPE: RNA
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<400> SEQUENCE: 57

guugccuccg guucugaagg uguuc 25

<210> SEQ ID NO 58
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<212> TYPE: RNA
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<400> SEQUENCE: 58

caacuguugc cuccgguucu gaagg 25

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<400> SEQUENCE: 59

ucauucaacu guugccuccg guucu 25

<210> SEQ ID NO 60
<211> LENGTH: 25
<212> TYPE: RNA
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<400> SEQUENCE: 60

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<210> SEQ ID NO 61
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

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<400> SEQUENCE: 61

cuuuaacauu ucauucaacu guugc 25

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<211> LENGTH: 25
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<400> SEQUENCE: 62

gaauccuuua acauuucauu caacu 25

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<400> SEQUENCE: 63

guguugaauc cuuuaacauu ucauu 25

<210> SEQ ID NO 64
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<400> SEQUENCE: 64

ccauuguguu gaauccuuua acauu 25

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<400> SEQUENCE: 65

uccagccauu guguugaauc cuuua 25

<210> SEQ ID NO 66
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<400> SEQUENCE: 66

uagcuuccag ccauuguguu gaauc 25

<210> SEQ ID NO 67
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<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 67

uuccuuagcu uccagccauu guguu 25

<210> SEQ ID NO 68

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<211> LENGTH: 25
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 68

gcuucuuccu uagcuuccag ccauu 25

<210> SEQ ID NO 69
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<220> FEATURE:
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<400> SEQUENCE: 69

gcucagcuuc uuccuuagcu uccag 25

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<400> SEQUENCE: 70

gaccugcuca gcuucuuccu uagcu 25

<210> SEQ ID NO 71
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<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 71

ccuaagaccu gcucagcuuc uuccu 25

<210> SEQ ID NO 72
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 72

ccuguccuaa gaccugcuca gcuuc 25

<210> SEQ ID NO 73
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 73

ucuggccugu ccuaagaccu gcuca 25

<210> SEQ ID NO 74
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 74

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uuggcucugg ccuguccuaa gaccu 25

<210> SEQ ID NO 75
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 75

caagcuuggc ucuggccugu ccuaa 25

<210> SEQ ID NO 76
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<212> TYPE: RNA
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<400> SEQUENCE: 76

ugacucaagc uuggcucugg ccugu 25

<210> SEQ ID NO 77
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<400> SEQUENCE: 77

uuccaugacu caagcuuggc ucugg 25

<210> SEQ ID NO 78
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<212> TYPE: RNA
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 78

ccuccuucca ugacucaagc uuggc 25

<210> SEQ ID NO 79
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 79

gggacccucc uuccaugacu caagc 25

<210> SEQ ID NO 80
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<212> TYPE: RNA
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

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cuguugccuc cgguucug 18

The invention claimed is:

1. An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of the nucleotide sequence of SEQ ID NO: 57, wherein the antisense oligomer is an oligonucleotide in which the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified, or a morpholino oligomer.

2. The antisense oligomer according to claim 1, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of: OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br, and I, wherein R is an alkyl or an aryl and R' is an alkylene.

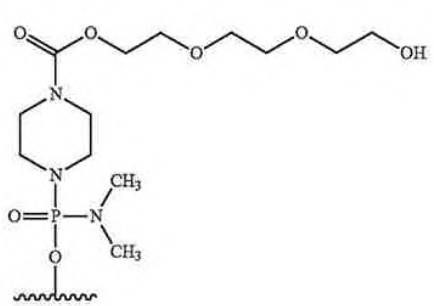
3. The antisense oligomer according to claim 1, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of: a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond, and a boranophosphate bond.

4. The antisense oligomer according to claim 1, which is a morpholino oligomer.

5. The antisense oligomer according to claim 4, which is a phosphorodiamidate morpholino oligomer.

6. The antisense oligomer according to claim 4, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:

(1)



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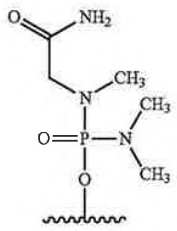

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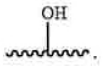

7. A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to claim 1, or a pharmaceutically acceptable salt or hydrate thereof.

* * * * *

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ISSUE DATE: *June 16, 2020*

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Certifying Officer

US010683322B2

(12) United States Patent

Watanabe et al.

(10) Patent No.: US 10,683,322 B2
(45) Date of Patent: *Jun. 16, 2020

(54) **ANTISENSE NUCLEIC ACIDS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **16/717,274**

(22) Filed: **Dec. 17, 2019**

(65) **Prior Publication Data**

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(63) Continuation of application No. 16/449,537, filed on Jun. 24, 2019, which is a continuation of application No. 15/619,996, filed on Jun. 12, 2017, now Pat. No. 10,329,319, which is a continuation of application No. 14/615,504, filed on Feb. 6, 2015, now Pat. No. 9,708,361, which is a continuation of application No. 13/819,520, filed as application No. PCT/JP2011/070318 on Aug. 31, 2011, now Pat. No. 9,079,934.

(30) **Foreign Application Priority Data**

Sep. 1, 2010 (JP) 2010-196032

(51) **Int. Cl.**

C12N 15/11	(2006.01)
C12N 15/113	(2010.01)
C07H 21/00	(2006.01)
C07H 21/04	(2006.01)
C12Q 1/68	(2018.01)

(52) **U.S. Cl.**

CPC ***C07H 21/04*** (2013.01); ***C07H 21/00*** (2013.01); ***C12N 15/111*** (2013.01); ***C12N 15/113*** (2013.01); *C12N 2310/11* (2013.01); *C12N 2310/315* (2013.01); *C12N 2310/3145* (2013.01); *C12N 2310/321* (2013.01); *C12N 2310/3525* (2013.01); *C12N 2320/33* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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Primary Examiner — Sean McGarry

(74) *Attorney, Agent, or Firm* — Faegre Drinker Biddle & Reath LLP

(57) **ABSTRACT**

The present invention provides an oligomer which efficiently enables to cause skipping of the 53rd exon in the human dystrophin gene. Also provided is a pharmaceutical composition which causes skipping of the 53rd exon in the human dystrophin gene with a high efficiency.

10 Claims, 19 Drawing Sheets

Specification includes a Sequence Listing.

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Figure 1

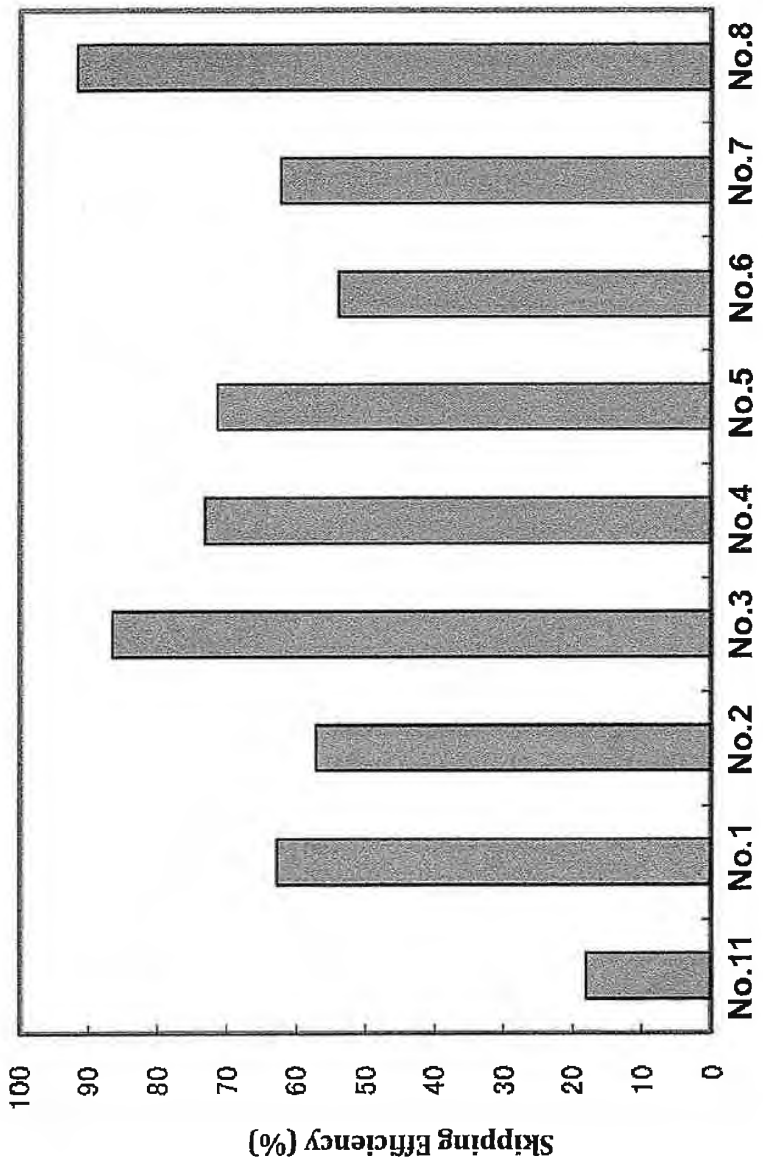

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Figure 2

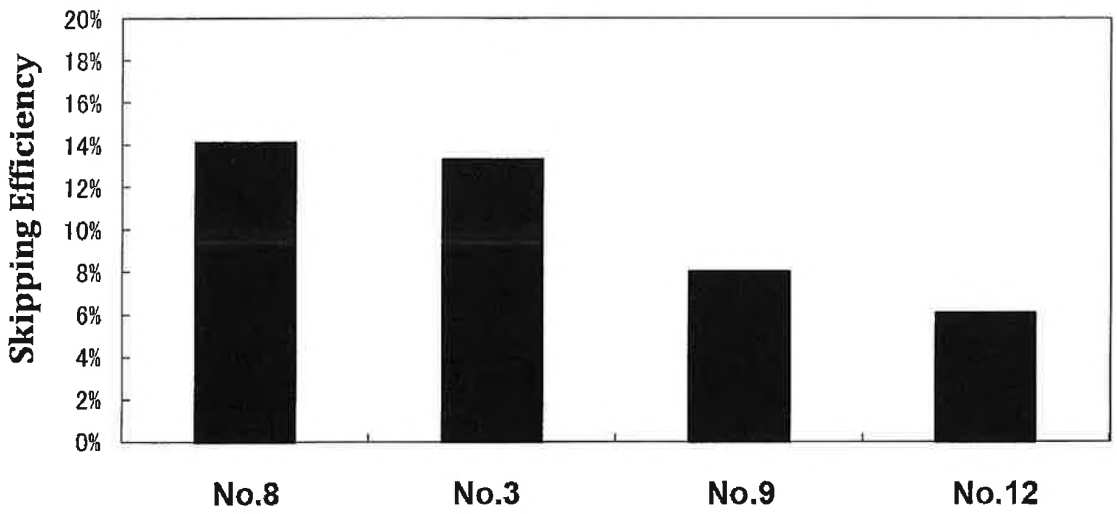

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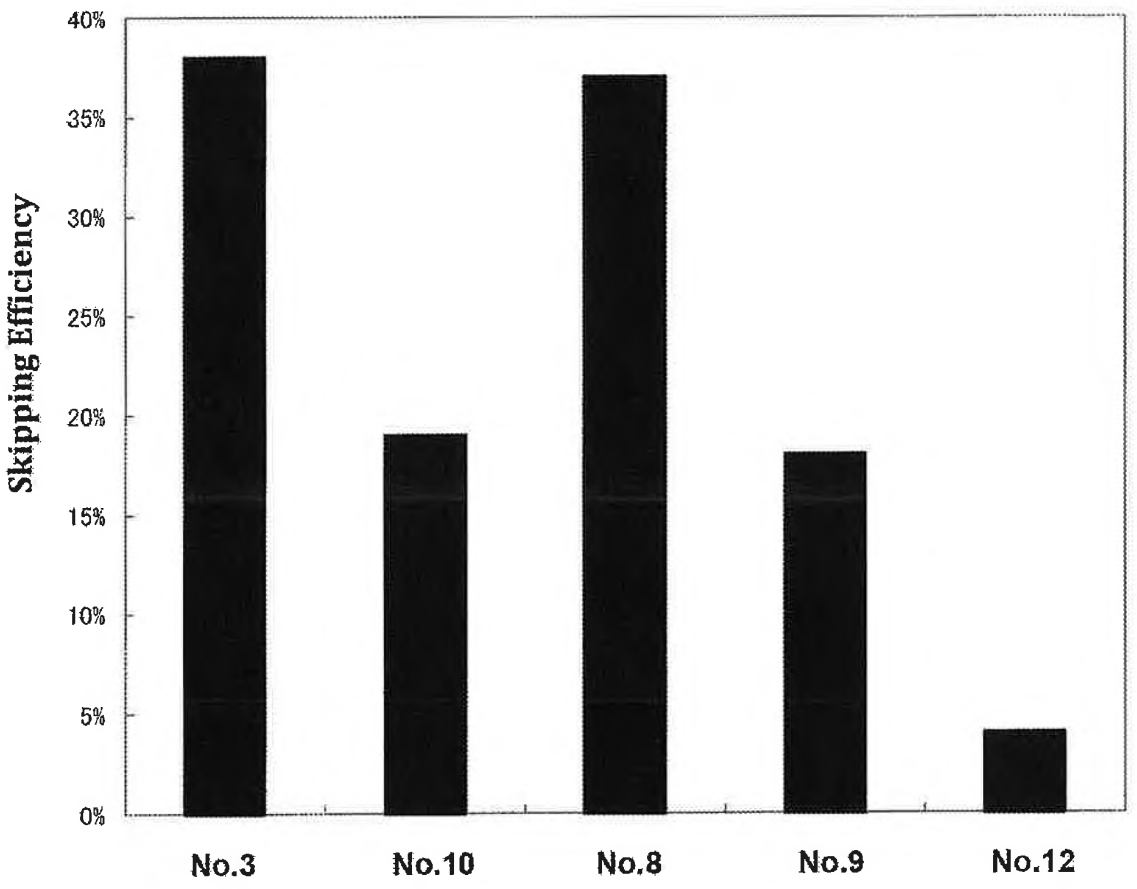
Figure 3



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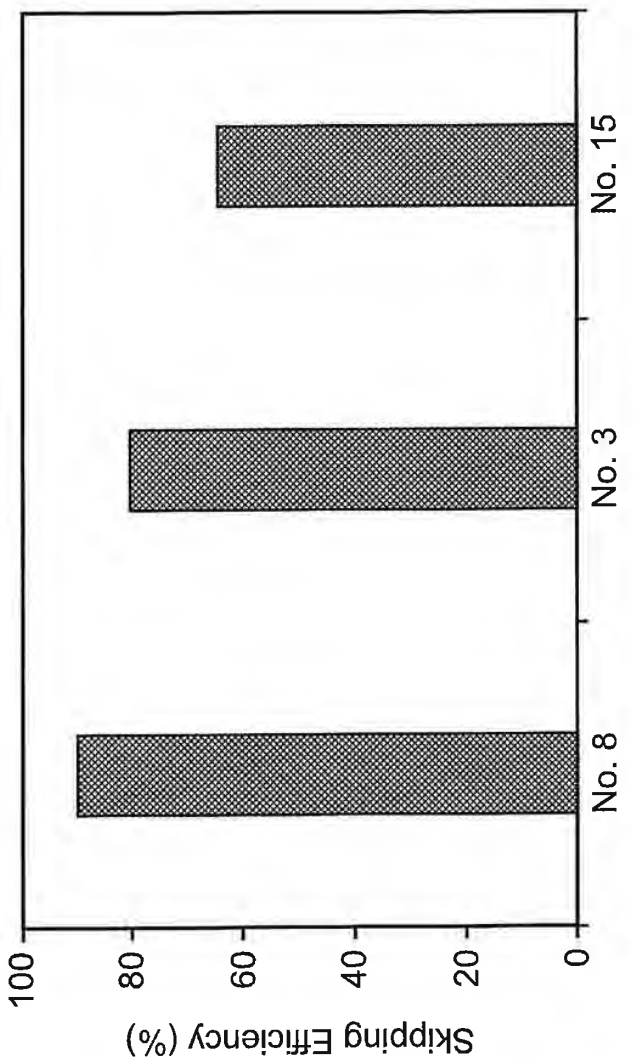

FIG. 4

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Figure 5

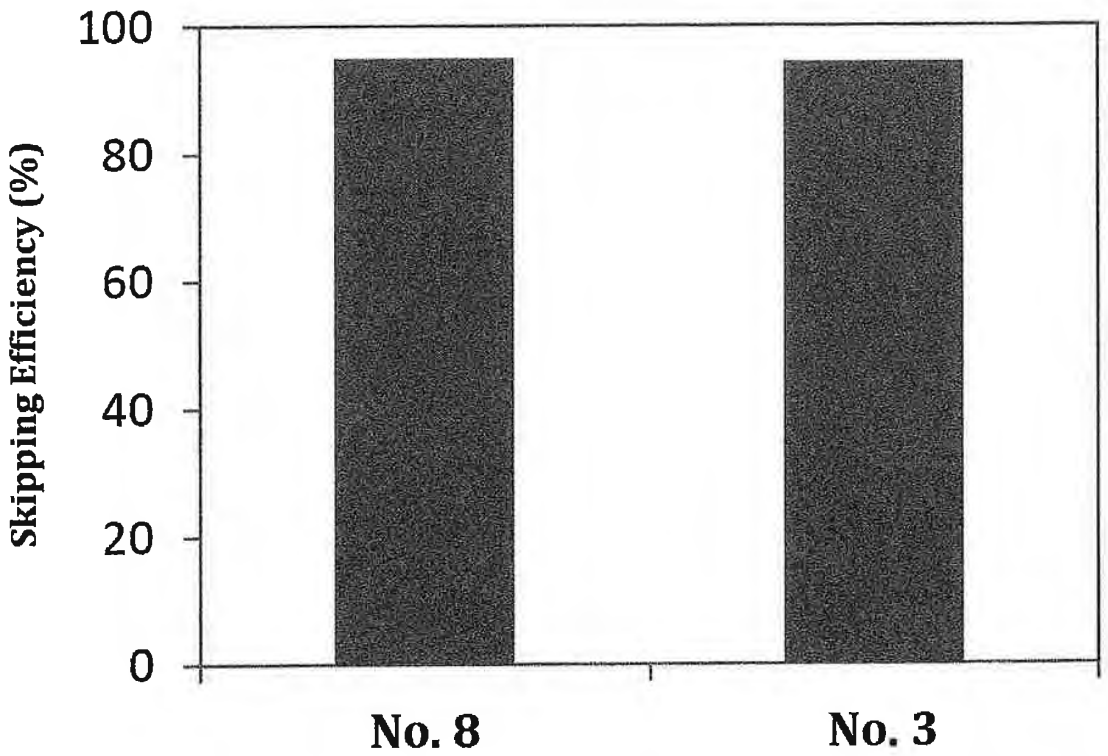

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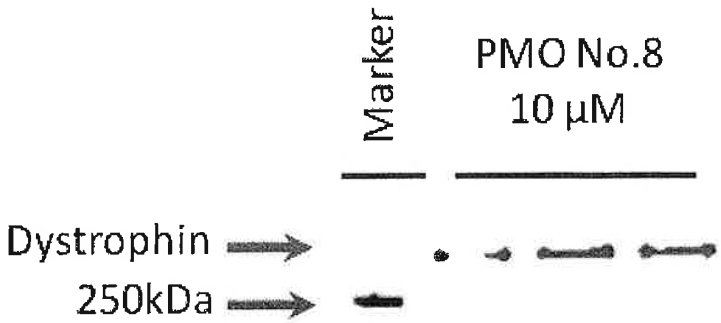
Figure 6



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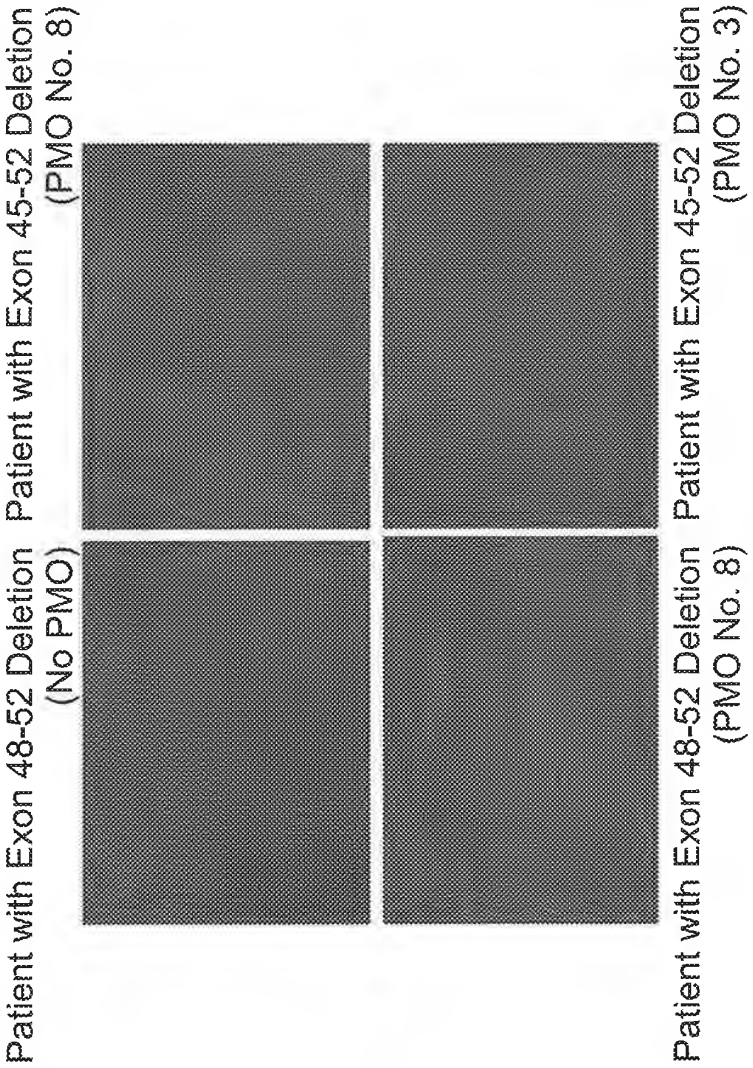

FIG. 7

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Figure 8

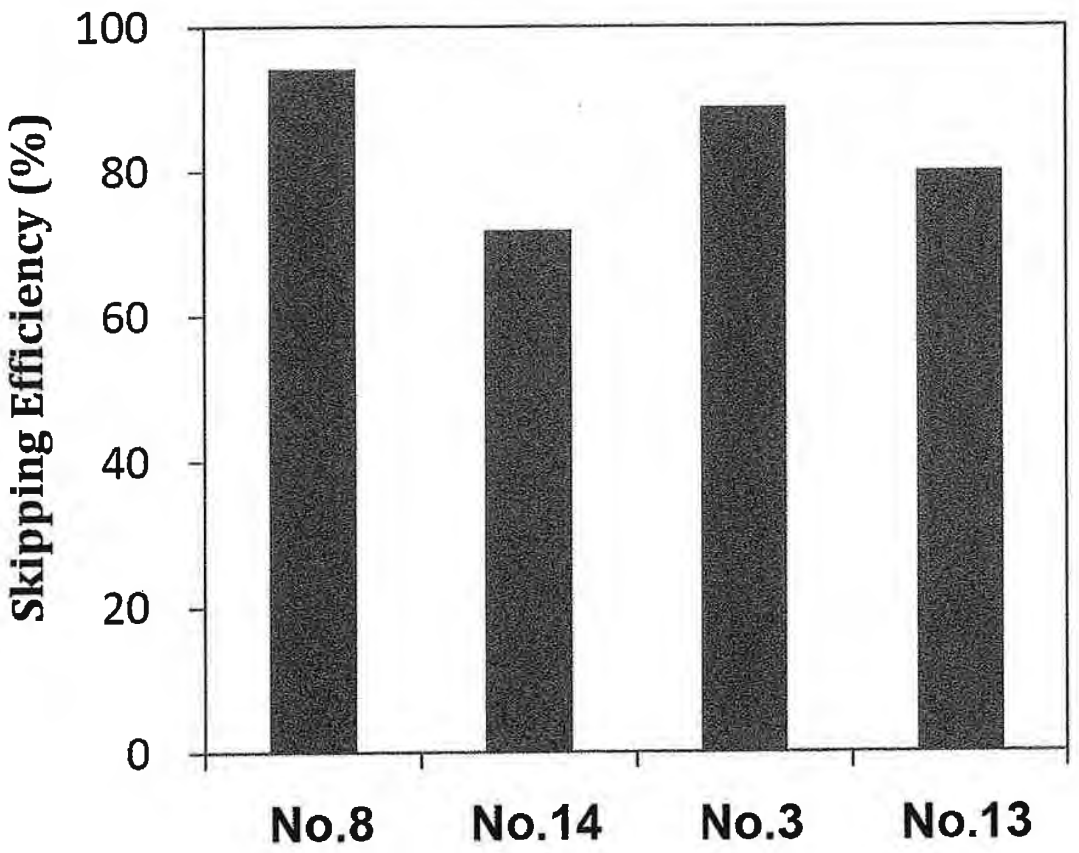

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FIG. 9

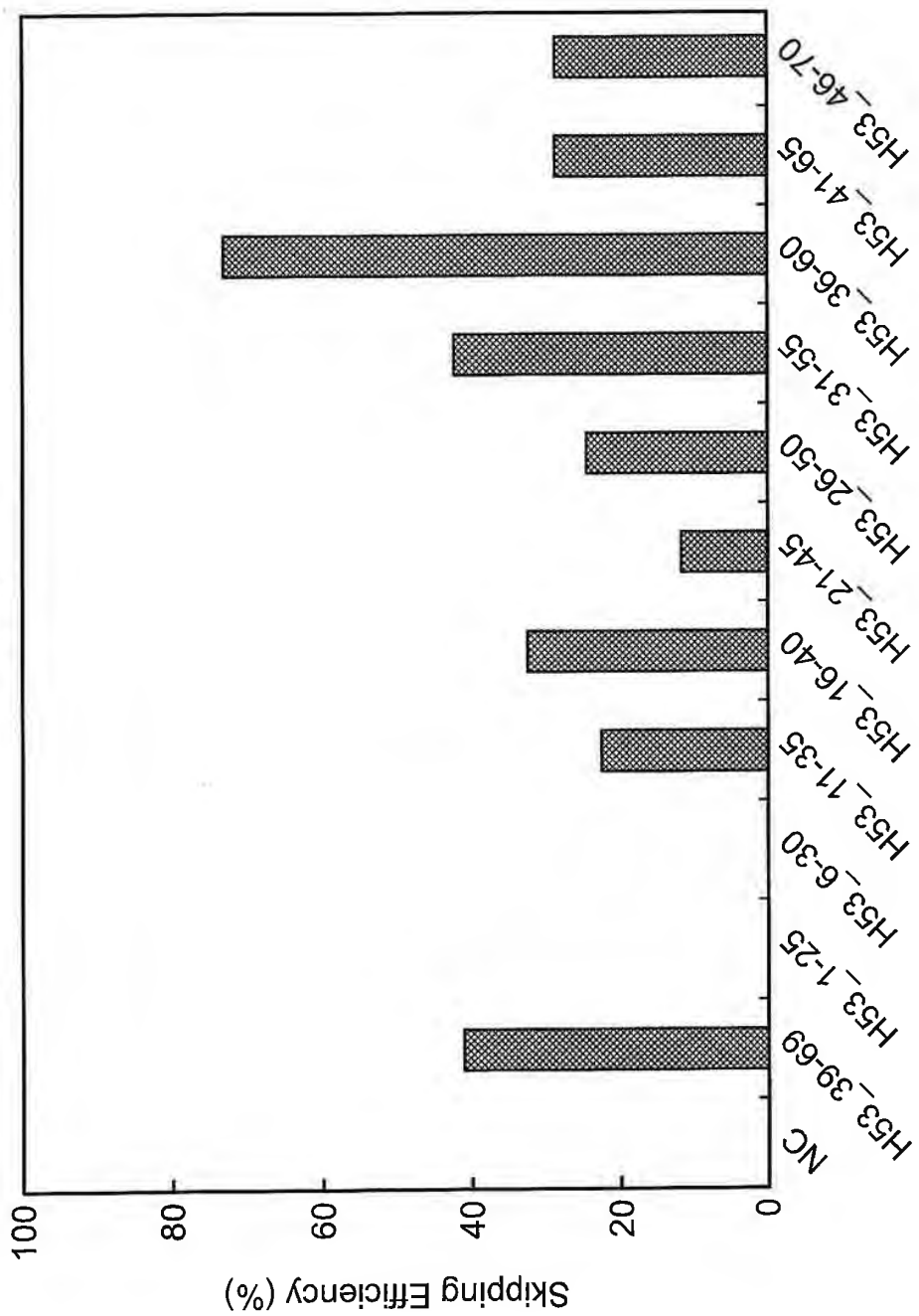

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FIG. 10

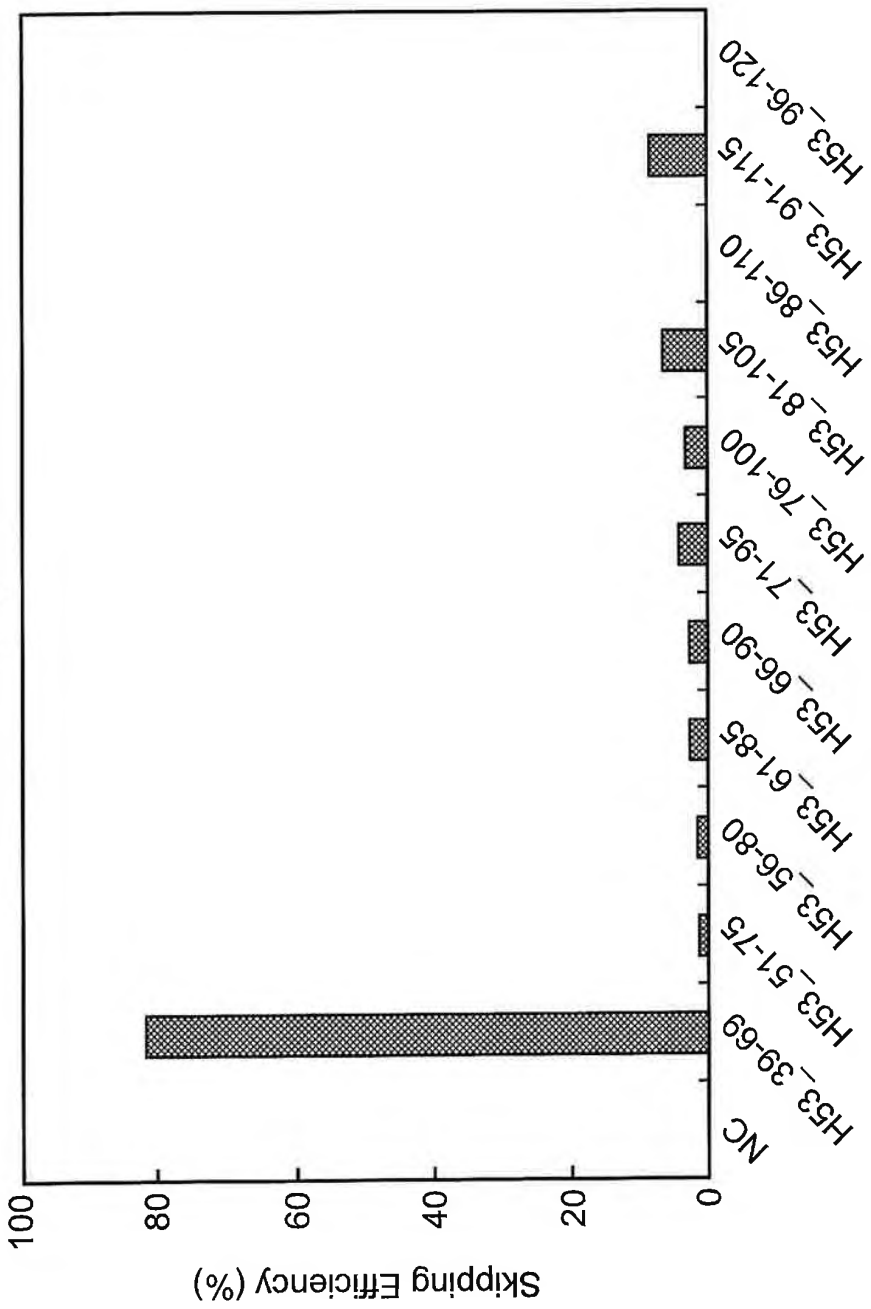

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FIG. 11

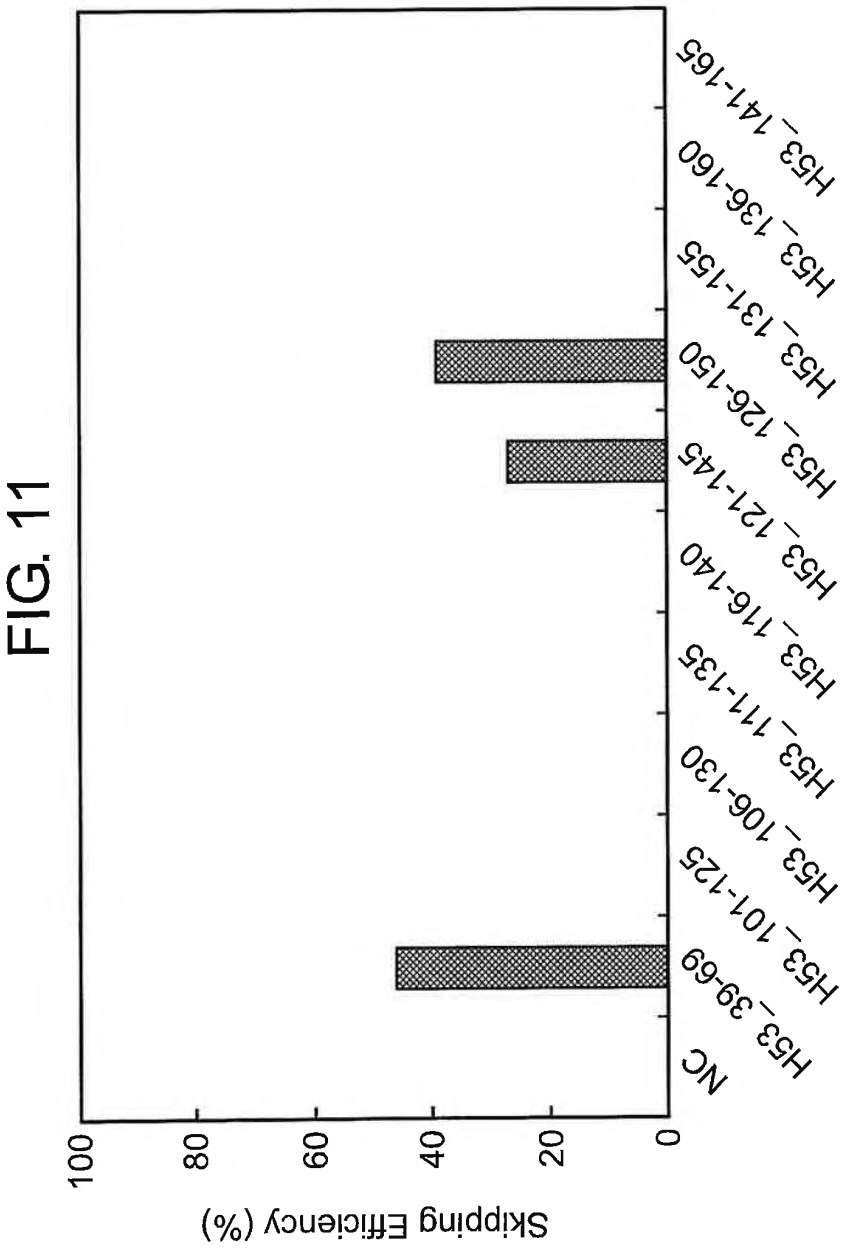

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FIG. 12

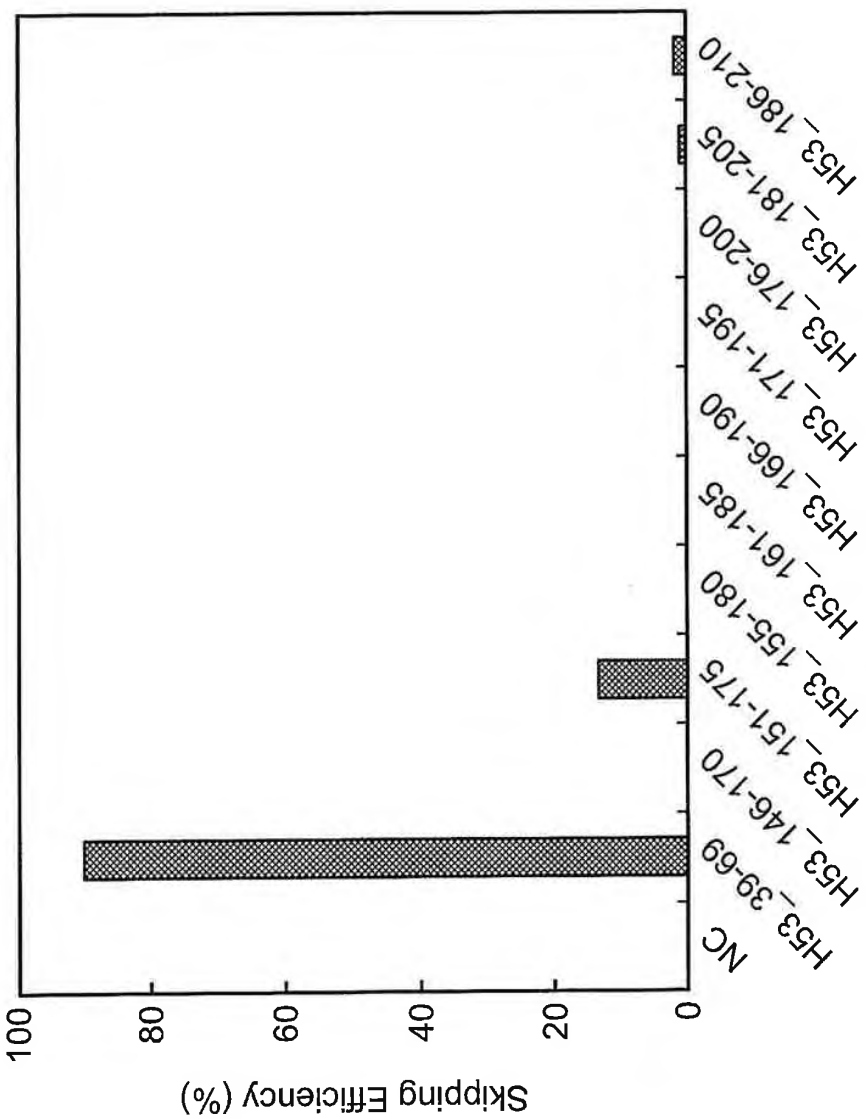

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FIG. 13

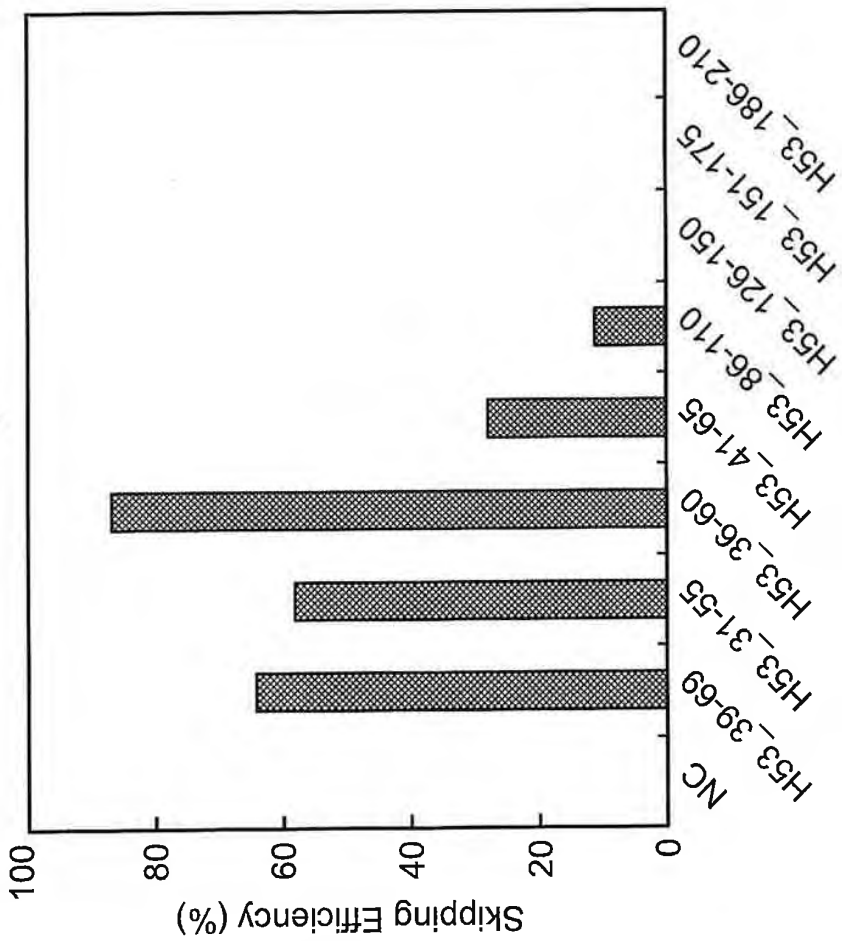

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FIG. 14

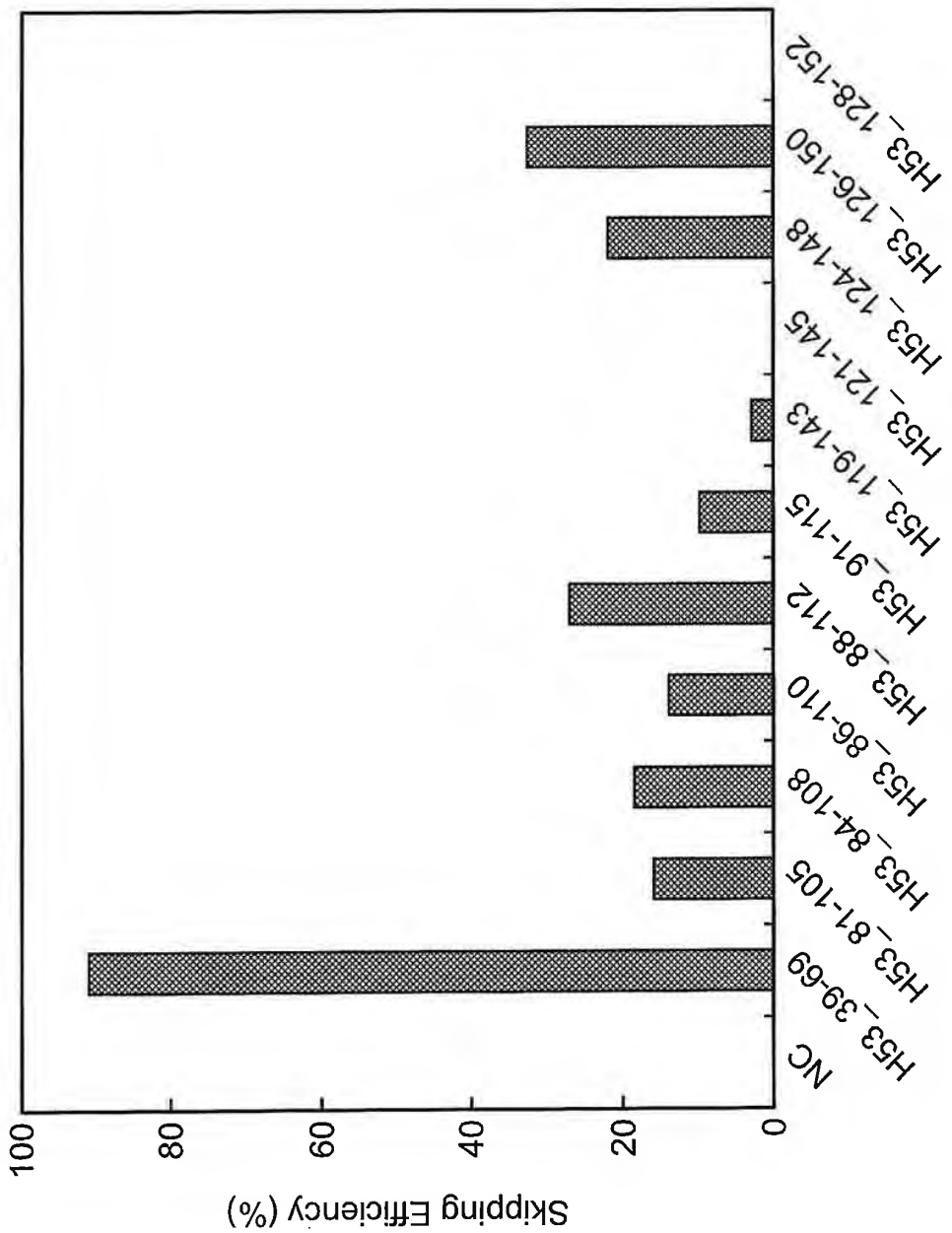

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FIG. 15

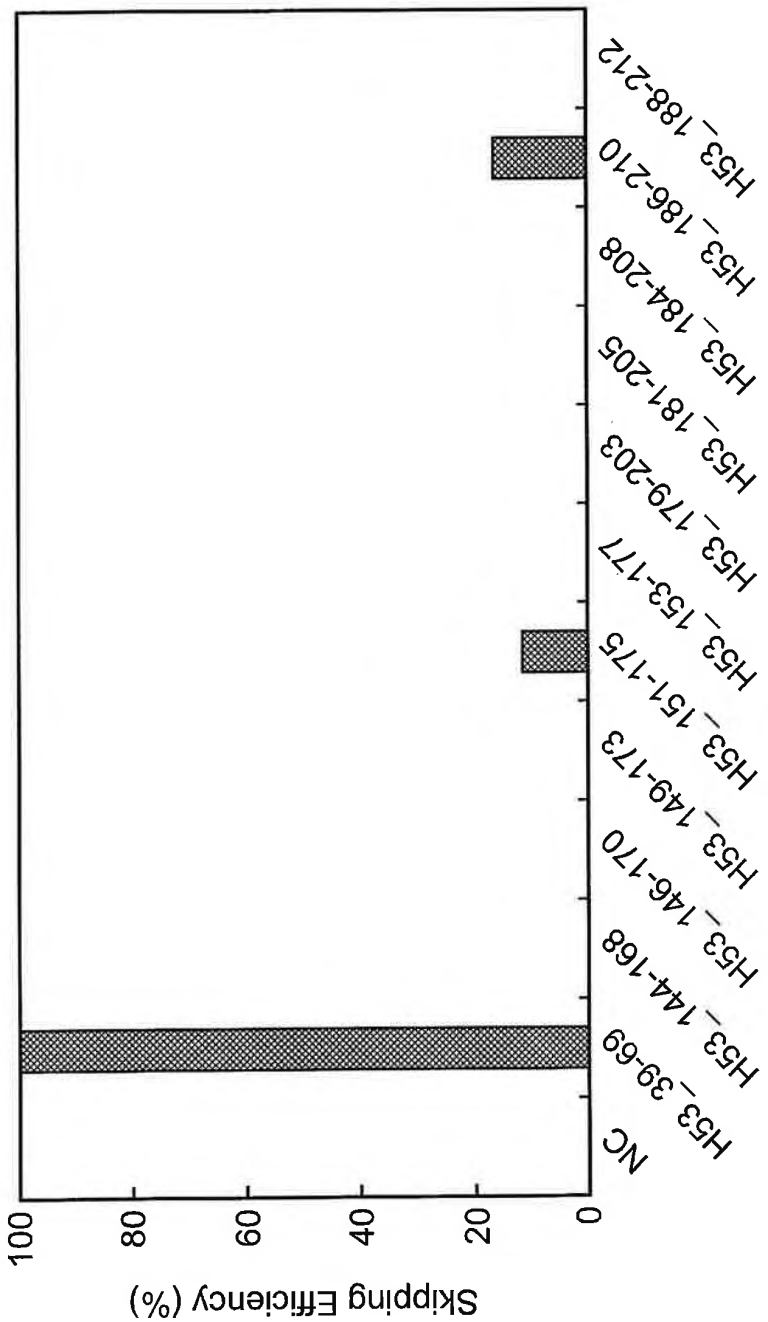

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FIG. 16

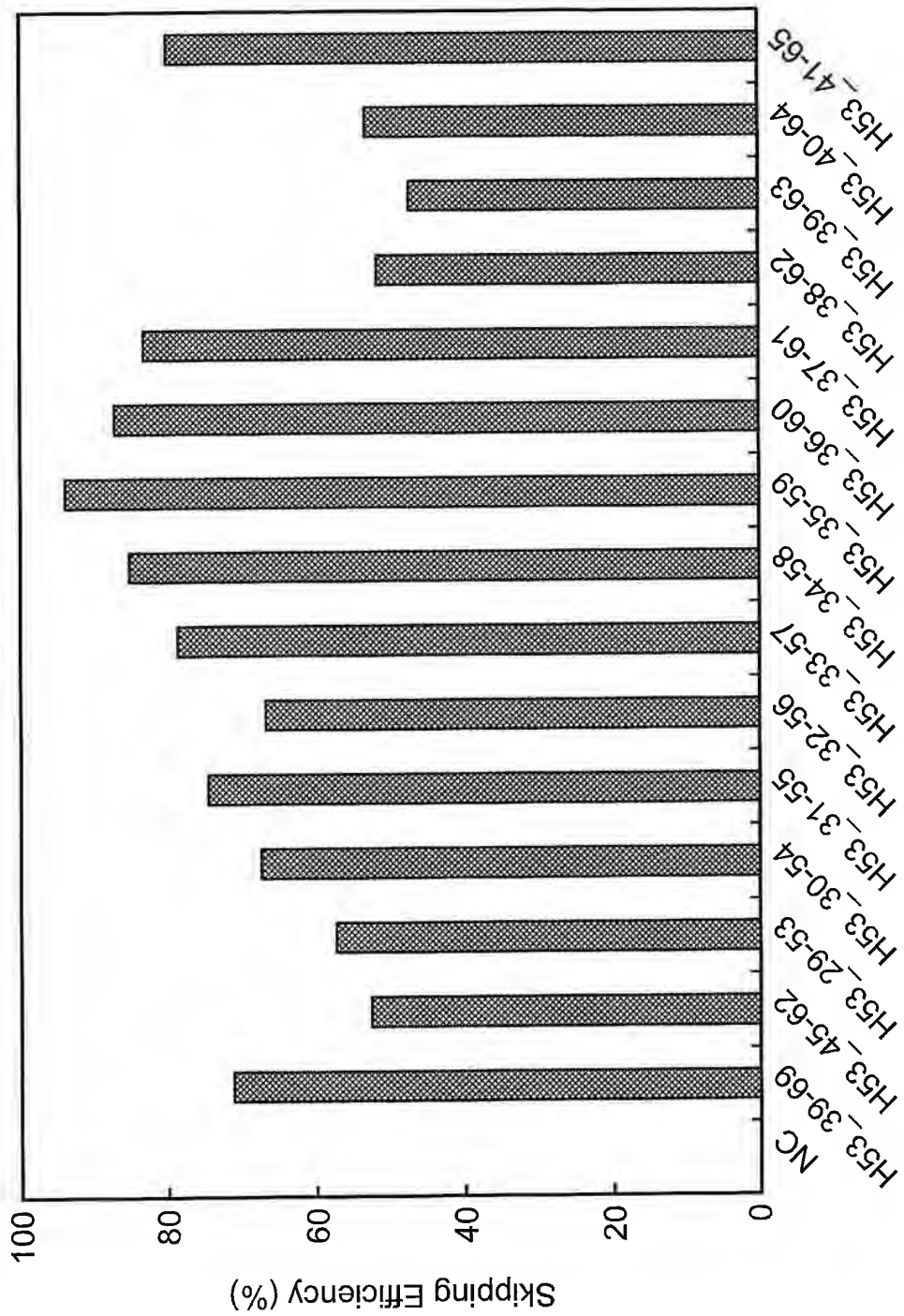

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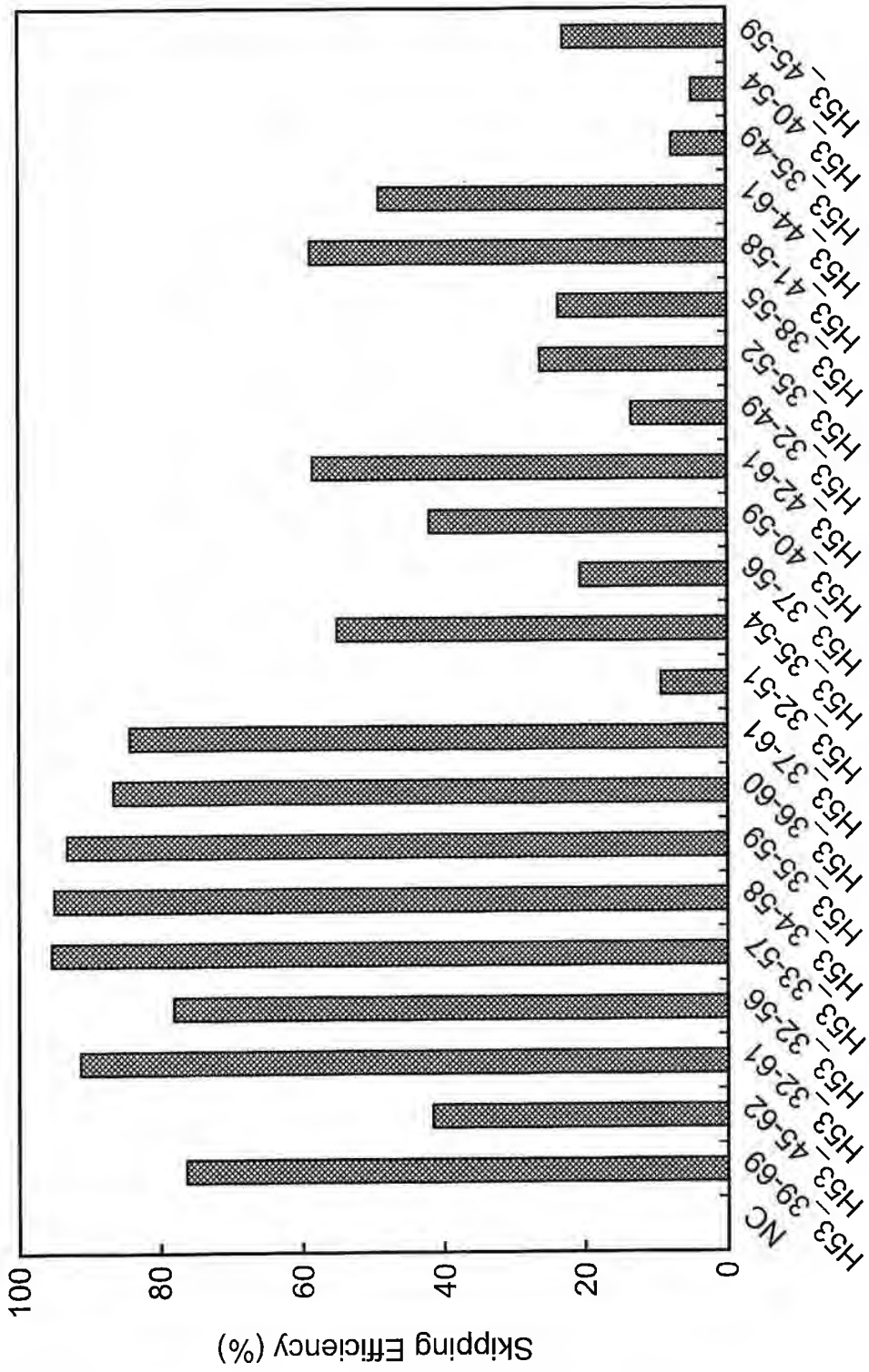
FIG. 17



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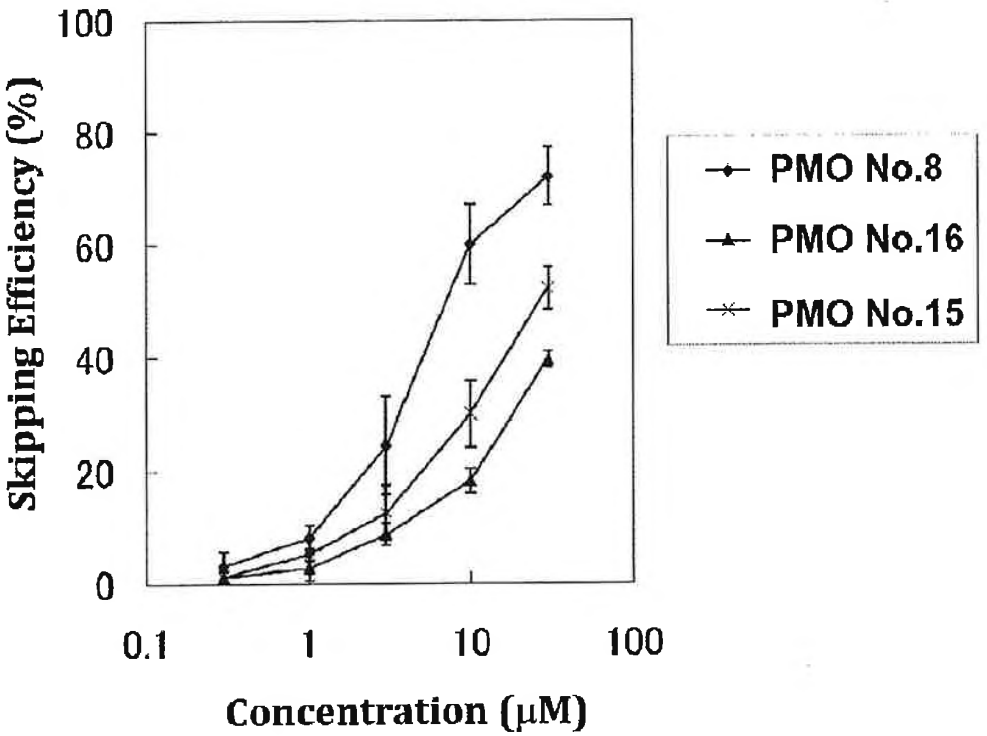

Figure 18

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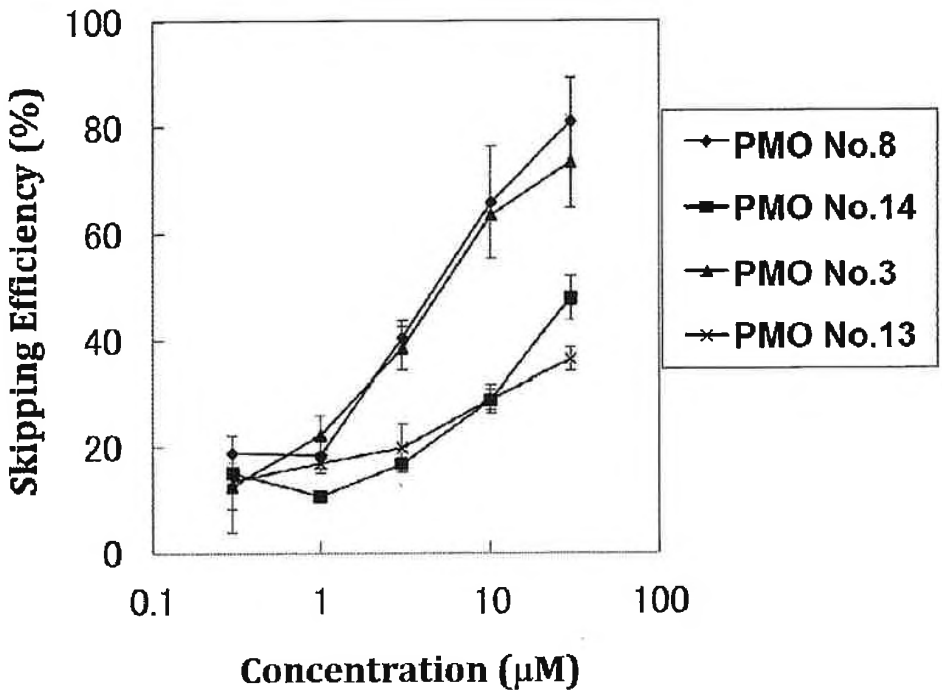

Figure 19

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ANTISENSE NUCLEIC ACIDS

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a Continuation of copending application Ser. No. 16/449,537 (allowed), filed Jun. 24, 2019, which is a Continuation of application Ser. No. 15/619,996, filed Jun. 12, 2017 (now U.S. Pat. No. 10,329,319 issued Jun. 25, 2019), which is a Continuation of application Ser. No. 14/615,504, filed Feb. 6, 2015 (now U.S. Pat. No. 9,708,361 issued Jul. 18, 2017), which is a Continuation of application Ser. No. 13/819,520, filed Apr. 10, 2013 (now U.S. Pat. No. 9,079,934 issued Jul. 14, 2015), which is a PCT National Stage of PCT/JP2011/070318 filed Aug. 31, 2011, which claims priority to JP Application No. 2010-196032 filed Sep. 1, 2010, all of which are incorporated by reference in their entireties.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Dec. 9, 2019 is named 209658_0001_09_US_594705_ST25.txt and is 24,765 bytes in size.

TECHNICAL FIELD

The present invention relates to an antisense oligomer which causes skipping of exon 53 in the human dystrophin gene, and a pharmaceutical composition comprising the oligomer.

BACKGROUND ART

Duchenne muscular dystrophy (DMD) is the most frequent form of hereditary progressive muscular dystrophy that affects one in about 3,500 newborn boys. Although the motor functions are rarely different from healthy humans in infancy and childhood, muscle weakness is observed in children from around 4 to 5 years old. Then, muscle weakness progresses to the loss of ambulation by about 12 years old and death due to cardiac or respiratory insufficiency in the twenties. DMD is such a severe disorder. At present, there is no effective therapy for DMD available, and it has been strongly desired to develop a novel therapeutic agent.

DMD is known to be caused by a mutation in the dystrophin gene. The dystrophin gene is located on X chromosome and is a huge gene consisting of 2.2 million DNA nucleotide pairs. DNA is transcribed into mRNA precursors, and introns are removed by splicing to synthesize mRNA in which 79 exons are joined together. This mRNA is translated into 3,685 amino acids to produce the dystrophin protein. The dystrophin protein is associated with the maintenance of membrane stability in muscle cells and necessary to make muscle cells less fragile. The dystrophin gene from patients with DMD contains a mutation and hence, the dystrophin protein, which is functional in muscle cells, is rarely expressed. Therefore, the structure of muscle cells cannot be maintained in the body of the patients with DMD, leading to a large influx of calcium ions into muscle cells. Consequently, an inflammation-like response occurs to promote fibrosis so that muscle cells can be regenerated only with difficulty.

Becker muscular dystrophy (BMD) is also caused by a mutation in the dystrophin gene. The symptoms involve muscle weakness accompanied by atrophy of muscle but are typically mild and slow in the progress of muscle weakness, when compared to DMD. In many cases, its onset is in adulthood. Differences in clinical symptoms between DMD and BMD are considered to reside in whether the reading frame for amino acids on the translation of dystrophin mRNA into the dystrophin protein is disrupted by the mutation or not (Non-Patent Document 1). More specifically, in DMD, the presence of mutation shifts the amino acid reading frame so that the expression of functional dystrophin protein is abolished, whereas in BMD the dystrophin protein that functions, though imperfectly, is produced because the amino acid reading frame is preserved, while a part of the exons are deleted by the mutation.

Exon skipping is expected to serve as a method for treating DMD. This method involves modifying splicing to restore the amino acid reading frame of dystrophin mRNA and induce expression of the dystrophin protein having the function partially restored (Non-Patent Document 2). The amino acid sequence part, which is a target for exon skipping, will be lost. For this reason, the dystrophin protein expressed by this treatment becomes shorter than normal one but since the amino acid reading frame is maintained, the function to stabilize muscle cells is partially retained. Consequently, it is expected that exon skipping will lead DMD to the similar symptoms to that of BMD which is milder. The exon skipping approach has passed the animal tests using mice or dogs and now is currently assessed in clinical trials on human DMD patients.

The skipping of an exon can be induced by binding of antisense nucleic acids targeting either 5' or 3' splice site or both sites, or exon-internal sites. An exon will only be included in the mRNA when both splice sites thereof are recognized by the spliceosome complex. Thus, exon skipping can be induced by targeting the splice sites with antisense nucleic acids. Furthermore, the binding of an SR protein to an exonic splicing enhancer (ESE) is considered necessary for an exon to be recognized by the splicing mechanism. Accordingly, exon skipping can also be induced by targeting ESE.

Since a mutation of the dystrophin gene may vary depending on DMD patients, antisense nucleic acids need to be designed based on the site or type of respective genetic mutation. In the past, antisense nucleic acids that induce exon skipping for all 79 exons were produced by Steve Wilton, et al., University of Western Australia (Non-Patent Document 3), and the antisense nucleic acids which induce exon skipping for 39 exons were produced by Annemieke Aartsma-Rus, et al., Netherlands (Non-Patent Document 4).

It is considered that approximately 8% of all DMD patients may be treated by skipping the 53rd exon (hereinafter referred to as "exon 53"). In recent years, a plurality of research organizations reported on the studies where exon 53 in the dystrophin gene was targeted for exon skipping (Patent Documents 1 to 4; Non-Patent Document 5). However, a technique for skipping exon 53 with a high efficiency has not yet been established.

Patent Document 1: International Publication WO 2006/000057

Patent Document 2: International Publication WO 2004/048570

Patent Document 3: US 2010/0168212

Patent Document 4: International Publication WO 2010/048586

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Non-Patent Document 1: Monaco A. P. et al., Genomics 1988; 2: p. 90-95
Non-Patent Document 2: Matsuo M., Brain Dev 1996; 18: p. 167-172
Non-Patent Document 3: Wilton S. D., e t al., Molecular Therapy 2007: 15: p. 1288-96
Non-Patent Document 4: Annemieke Aartsma-Rus et al., (2002) Neuromuscular Disorders 12: S71-S77
Non-Patent Document 5: Linda J. Popplewell et al., (2010) Neuromuscular Disorders, vol. 20, no. 2, p. 102-10

DISCLOSURE OF THE INVENTION

Under the foregoing circumstances, antisense oligomers that strongly induce exon 53 skipping in the dystrophin gene and muscular dystrophy therapeutics comprising oligomers thereof have been desired.

As a result of detailed studies of the structure of the dystrophin gene, the present inventors have found that exon 53 skipping can be induced with a high efficiency by targeting the sequence consisting of the 32nd to the 56th nucleotides from the 5' end of exon 53 in the mRNA precursor (hereinafter referred to as "pre-mRNA") in the dystrophin gene with antisense oligomers. Based on this finding, the present inventors have accomplished the present invention.

That is, the present invention is as follows.

[1] An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences consisting of the 31st to the 53rd, the 31st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.

[2] The antisense oligomer according to [1] above, which is an oligonucleotide.

[3] The antisense oligomer according to [2] above, wherein the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.

[4] The antisense oligomer according to [3] above, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br and I (wherein R is an alkyl or an aryl and R' is an alkylene).

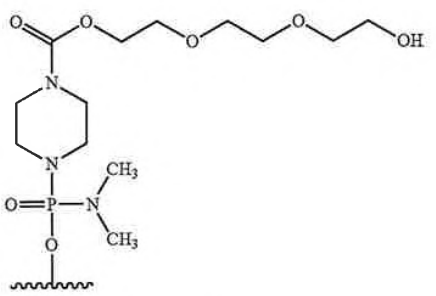
[5] The antisense oligomer according to [3] or [4] above, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond and a boranophosphate bond.

[6] The antisense oligomer according to [1] above, which is a morpholino oligomer.

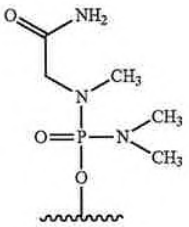
[7] The antisense oligomer according to [6] above, which is a phosphorodiamidate morpholino oligomer.

[8] The antisense oligomer according to any one of [1] to [7] above, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:

(1)



(2)



(3)

[9] The antisense oligomer according to any one of [1] to [8] above, consisting of a nucleotide sequence complementary to the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene.

[10] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 2 to 37.

[11] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 11, 17, 23, 29 and 35.

[12] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by SEQ ID NO: 11 or 35.

[13] A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to any one of [1] to [12] above, or a pharmaceutically acceptable salt or hydrate thereof.

The antisense oligomer of the present invention can induce exon 53 skipping in the human dystrophin gene with a high efficiency. In addition, the symptoms of Duchenne muscular dystrophy can be effectively alleviated by administering the pharmaceutical composition of the present invention.

BRIEF DESCRIPTION OF DRAWINGS

FIG. **1** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cell line (RD cells).

FIG. **2** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD

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gene is introduced into human normal tissue-derived fibroblasts (TIG-119 cells) to induce differentiation into muscle cells.

FIG. **3** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human DMD patient-derived fibroblasts (5017 cells) to induce differentiation into muscle cells.

FIG. **4** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. **5** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. **6** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. **7** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52 or deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. **8** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. **9** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **10** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **11** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **12** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **13** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **14** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **15** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **16** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **17** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **18** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

FIG. **19** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention is described in detail. The embodiments described below are intended to be presented by way of example merely to describe the invention but not limited only to the following embodiments. The present invention may be implemented in various ways without departing from the gist of the invention.

All of the publications, published patent applications, patents and other patent documents cited in the specification are herein incorporated by reference in their entirety. The specification hereby incorporates by reference the contents of the specification and drawings in the Japanese Patent Application (No. 2010-196032) filed Sep. 1, 2010, from which the priority was claimed.

1. Antisense Oligomer

The present invention provides the antisense oligomer (hereinafter referred to as the "oligomer of the present invention") which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences (hereinafter also referred to as "target sequences") consisting of the 31st to the 53rd, the 31st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.

[Exon 53 in Human Dystrophin Gene]

In the present invention, the term "gene" is intended to mean a genomic gene and also include cDNA, mRNA precursor and mRNA. Preferably, the gene is mRNA precursor, i.e., pre-mRNA.

In the human genome, the human dystrophin gene locates at locus Xp21.2. The human dystrophin gene has a size of 3.0 Mbp and is the largest gene among known human genes. However, the coding regions of the human dystrophin gene are only 14 kb, distributed as 79 exons throughout the human dystrophin gene (Roberts, R G., et al., Genomics, 16: 536-538 (1993)). The pre-mRNA, which is the transcript of the human dystrophin gene, undergoes splicing to generate mature mRNA of 14 kb. The nucleotide sequence of human wild-type dystrophin gene is known (GenBank Accession No. NM_004006).

The nucleotide sequence of exon 53 in the human wild-type dystrophin gene is represented by SEQ ID NO: 1.

The oligomer of the present invention is designed to cause skipping of exon 53 in the human dystrophin gene, thereby modifying the protein encoded by DMD type of dystrophin gene into the BMD type of dystrophin protein. Accordingly, exon 53 in the dystrophin gene that is the target of exon skipping by the oligomer of the present invention includes both wild and mutant types.

Specifically, exon 53 mutants of the human dystrophin gene include the polynucleotides defined in (a) or (b) below.

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(a) A polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1; and,

(b) A polynucleotide consisting of a nucleotide sequence having at least 90% identity with the nucleotide sequence of SEQ ID NO: 1.

As used herein, the term "polynucleotide" is intended to mean DNA or RNA.

As used herein, the term "polynucleotide that hybridizes under stringent conditions" refers to, for example, a polynucleotide obtained by colony hybridization, plaque hybridization, Southern hybridization or the like, using as a probe all or part of a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of, e.g., SEQ ID NO: 1. The hybridization method which may be used includes methods described in, for example, "Sambrook & Russell, Molecular Cloning: A Laboratory Manual Vol. 3, Cold Spring Harbor, Laboratory Press 2001," "Ausubel, Current Protocols in Molecular Biology, John Wiley & Sons 1987-1997," etc.

As used herein, the term "complementary nucleotide sequence" is not limited only to nucleotide sequences that form Watson-Crick pairs with target nucleotide sequences, but is intended to also include nucleotide sequences which form Wobble base pairs. As used herein, the term Watson-Crick pair refers to a pair of nucleobases in which hydrogen bonds are formed between adenine-thymine, adenine-uracil or guanine-cytosine, and the term Wobble base pair refers to a pair of nucleobases in which hydrogen bonds are formed between guanine-uracil, inosine-uracil, inosine-adenine or inosine-cytosine. As used herein, the term "complementary nucleotide sequence" does not only refers to a nucleotide sequence 100% complementary to the target nucleotide sequence but also refers to a complementary nucleotide sequence that may contain, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target nucleotide sequence.

As used herein, the term "stringent conditions" may be any of low stringent conditions, moderate stringent conditions or high stringent conditions. The term "low stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 32° C. The term "moderate stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 42° C., or 5×SSC, 1% SDS, 50 mM Tris-HCl (pH 7.5), 50% formamide at 42° C. The term "high stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 50° C. or 0.2×SSC, 0.1% SDS at 65° C. Under these conditions, polynucleotides with higher homology are expected to be obtained efficiently at higher temperatures, although multiple factors are involved in hybridization stringency including temperature, probe concentration, probe length, ionic strength, time, salt concentration and others, and those skilled in the art may appropriately select these factors to achieve similar stringency.

When commercially available kits are used for hybridization, for example, an Alkphos Direct Labeling and Detection System (GE Healthcare) may be used. In this case, according to the attached protocol, after cultivation with a labeled probe overnight, the membrane is washed with a primary wash buffer containing 0.1% (w/v) SDS at 55° C., thereby detecting hybridized polynucleotides. Alternatively, in producing a probe based on the entire or part of the nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1, hybridization can be detected with a DIG Nucleic Acid Detection Kit (Roche Diagnostics) when the probe is labeled with digoxigenin (DIG) using a commercially available reagent (e.g., a PCR Labeling Mix (Roche Diagnostics), etc.).

In addition to the polynucleotides described above, other polynucleotides that can be hybridized include polynucleotides having 90% or higher, 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, 98% or higher, 99% or higher, 99.1% or higher, 99.2% or higher, 99.3% or higher, 99.4% or higher, 99.5% or higher, 99.6% or higher, 99.7% or higher, 99.8% or higher or 99.9% or higher identity with the polynucleotide of SEQ ID NO: 1, as calculated by homology search software BLAST using the default parameters.

The identity between nucleotide sequences may be determined using algorithm BLAST (Basic Local Alignment Search Tool) by Karlin and Altschul (Proc. Natl. Acad. Sci. USA 872264-2268, 1990; Proc. Natl. Acad. Sci. USA 90: 5873, 1993). Programs called BLASTN and BLASTX based on the BLAST algorithm have been developed (Altschul S F, et al: J. Mol. Biol. 215: 403, 1990). When a nucleotide sequence is sequenced using BLASTN, the parameters are, for example, score=100 and wordlength=12. When BLAST and Gapped BLAST programs are used, the default parameters for each program are employed.

Examples of the nucleotide sequences complementary to the sequences consisting of the 31st to the 53rd, the 31st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th and the 36th to the 58th nucleotides, from the 5' end of exon 53.

TABLE 1

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
31-53	5'-CCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 2
31-54	5'-TCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 3
31-55	5'-CTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 4
31-56	5'-CCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 5
31-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 6
31-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 7
32-53	5'-CCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 8
32-54	5'-TCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 9

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TABLE 1-continued

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
32-55	5'-CTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 10
32-56	5'-CCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 11
32-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 12
32-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 13
33-53	5'-CCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 14
33-54	5'-TCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 15
33-55	5'-CTCCGGTTCTGAAGGTGTTTCTTG-3'	SEQ ID NO: 16
33-56	5'-CCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 17
33-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 18
33-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 19
34-53	5'-CCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 20
34-54	5'-TCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 21
34-55	5'-CTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 22
34-56	5'-CCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 23
34-57	5'-GCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 24
34-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 25
35-53	5'-CCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 26
35-54	5'-TCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 27
35-55	5'-CTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 28
35-56	5'-CCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 29
35-57	5'-GCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 30
35-58	5'-TGCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 31
36-53	5'-CCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 32
36-54	5'-TCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 33
36-55	5'-CTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 34
36-56	5'-CCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 35
36-57	5'-GCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 36
36-58	5'-TGCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 37

It is preferred that the oligomer of the present invention consists of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th, the 33rd to the 56th, the 34th to the 56th, the 35th to the 56th or the 36th to the 56th nucleotides (e.g., SEQ ID NO: 11, SEQ ID NO: 17, SEQ ID NO: 23, SEQ ID NO: 29 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

Preferably, the oligomer of the present invention consists of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides (e.g., SEQ ID NO: 11 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

The term "cause skipping of the 53rd exon in the human dystrophin gene" is intended to mean that by binding of the oligomer of the present invention to the site corresponding to exon 53 of the transcript (e.g., pre-mRNA) of the human dystrophin gene, for example, the nucleotide sequence corresponding to the 5' end of exon 54 is spliced at the 3' side of the nucleotide sequence corresponding to the 3' end of exon 51 in DMD patients with deletion of, exon 52 when the transcript undergoes splicing, thus resulting in formation of mature mRNA which is free of codon frame shift.

Accordingly, it is not required for the oligomer of the present invention to have a nucleotide sequence 100% complementary to the target sequence, as far as it causes exon 53 skipping in the human dystrophin gene. The oligomer of the present invention may include, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target sequence.

Herein, the term "binding" described above is intended to mean that when the oligomer of the present invention is mixed with the transcript of human dystrophin gene, both are hybridized under physiological conditions to form a double strand nucleic acid. The term "under physiological conditions" refers to conditions set to mimic the in vivo environment in terms of pH, salt composition and temperature. The conditions are, for example, 25 to 40° C., preferably 37° C., pH 5 to 8, preferably pH 7.4 and 150 mM of sodium chloride concentration.

Whether the skipping of exon 53 in the human dystrophin gene is caused or not can be confirmed by introducing the oligomer of the present invention into a dystrophin expression cell (e.g., human rhabdomyosarcoma cells), amplifying the region surrounding exon 53 of mRNA of the human dystrophin gene from the total RNA of the dystrophin expression cell by RT-PCR and performing nested PCR or sequence analysis on the PCR amplified product.

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The skipping efficiency can be determined as follows. The mRNA for the human dystrophin gene is collected from test cells; in the mRNA, the polynucleotide level "A" of the band where exon 53 is skipped and the polynucleotide level "B" of the band where exon 53 is not skipped are measured. Using these measurement values of "A" and "B," the efficiency is calculated by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A+B) \times 100$$

The oligomer of the present invention includes, for example, an oligonucleotide, morpholino oligomer or peptide nucleic acid (PNA), having a length of 18 to 28 nucleotides. The length is preferably from 21 to 25 nucleotides and morpholino oligomers are preferred.

The oligonucleotide described above (hereinafter referred to as "the oligonucleotide of the present invention") is the oligomer of the present invention composed of nucleotides as constituent units. Such nucleotides may be any of ribonucleotides, deoxyribonucleotides and modified nucleotides.

The modified nucleotide refers to one having fully or partly modified nucleobases, sugar moieties and/or phosphate-binding regions, which constitute the ribonucleotide or deoxyribonucleotide.

The nucleobase includes, for example, adenine, guanine, hypoxanthine, cytosine, thymine, uracil, and modified bases thereof. Examples of such modified nucleobases include, but not limited to, pseudouracil, 3-methyluracil, dihydrouracil, 5-alkylcytosines (e.g., 5-methylcytosine), 5-alkyluracils (e.g., 5-ethyluracil), 5-halouracils (5-bromouracil), 6-azapyrimidine, 6-alkylpyrimidines (6-methyluracil), 2-thiouracil, 4-thiouracil, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5'-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, 1-methyladenine, 1-methylhypoxanthine, 2,2-dimethylguanine, 3-methylcytosine, 2-methyladenine, 2-methylguanine, N6-methyladenine, 7-methylguanine, 5-methoxyaminomethyl-2-thiouracil, 5-methylaminomethyluracil, 5-methylcarbonylmethyluracil, 5-methyloxyuracil, 5-methyl-2-thiouracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid, 2-thiocytosine, purine, 2,6-diaminopurine, 2-aminopurine, isoguanine, indole, imidazole, xanthine, etc.

Modification of the sugar moiety may include, for example, modifications at the 2'-position of ribose and modifications of the other positions of the sugar. The modification at the 2'-position of ribose includes replacement of the 2'-OH of ribose with OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br or I, wherein R represents an alkyl or an aryl and R' represents an alkylene.

The modification for the other positions of the sugar includes, for example, replacement of O at the 4' position of ribose or deoxyribose with S, bridging between 2' and 4' positions of the sugar, e.g., LNA (locked nucleic acid) or ENA (2'-O,4'-C-ethylene-bridged nucleic acids), but is not limited thereto.

A modification of the phosphate-binding region includes, for example, a modification of replacing phosphodiester bond with phosphorothioate bond, phosphorodithioate bond, alkyl phosphonate bond, phosphoroamidate bond or boranophosphate bond (Enya et al: Bioorganic & Medicinal Chemistry, 2008, 18, 9154-9160) (cf., e.g., Japan Domestic Re-Publications of PCT Application Nos. 2006/129594 and 2006/038608).

The alkyl is preferably a straight or branched alkyl having 1 to 6 carbon atoms. Specific examples include methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, n-pentyl, isopentyl, neopentyl, tert-pentyl, n-hexyl and isohexyl. The alkyl may optionally be substituted. Examples of such substituents are a halogen, an alkoxy, cyano and nitro. The alkyl may be substituted with 1 to 3 substituents.

The cycloalkyl is preferably a cycloalkyl having 5 to 12 carbon atoms. Specific examples include cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, cyclodecyl and cyclododecyl.

The halogen includes fluorine, chlorine, bromine and iodine.

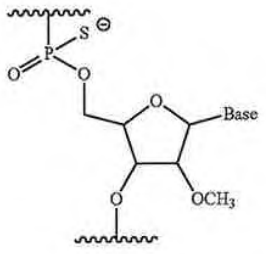
The alkoxy is a straight or branched alkoxy having 1 to 6 carbon atoms such as methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, isobutoxy, sec-butoxy, tert-butoxy, n-pentyloxy, isopentyloxy, n-hexyloxy, isohexyloxy, etc. Among others, an alkoxy having 1 to 3 carbon atoms is preferred.

The aryl is preferably an aryl having 6 to 10 carbon atoms. Specific examples include phenyl, α-naphthyl and β-naphthyl. Among others, phenyl is preferred. The aryl may optionally be substituted. Examples of such substituents are an alkyl, a halogen, an alkoxy, cyano and nitro. The aryl may be substituted with one to three of such substituents.

The alkylene is preferably a straight or branched alkylene having 1 to 6 carbon atoms. Specific examples include methylene, ethylene, trimethylene, tetramethylene, pentamethylene, hexamethylene, 2-(ethyl) trimethylene and 1-(methyl) tetramethylene.

The acyl includes a straight or branched alkanoyl or aroyl. Examples of the alkanoyl include formyl, acetyl, 2-methylacetyl, 2,2-dimethylacetyl, propionyl, butyryl, isobutyryl, pentanoyl, 2,2-dimethylpropionyl, hexanoyl, etc. Examples of the aroyl include benzoyl, toluoyl and naphthoyl. The aroyl may optionally be substituted at substitutable positions and may be substituted with an alkyl(s).

Preferably, the oligonucleotide of the present invention is the oligomer of the present invention containing a constituent unit represented by general formula below wherein the —OH group at position 2' of ribose is substituted with methoxy and the phosphate-binding region is a phosphorothioate bond:



wherein Base represents a nucleobase.

The oligonucleotide of the present invention may be easily synthesized using various automated synthesizer (e.g., AKTA oligopilot plus 10/100 (GE Healthcare)). Alternatively, the synthesis may also be entrusted to a third-party organization (e.g., Promega Inc., or Takara Co.), etc.

The morpholino oligomer of the present invention is the oligomer of the present invention comprising the constituent unit represented by general formula below:

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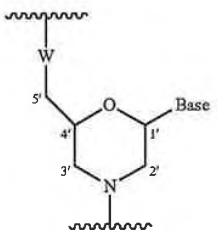
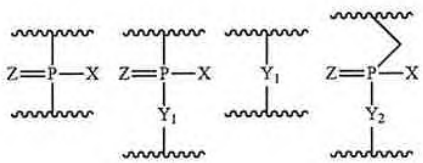


wherein Base has the same significance as defined above, and,

W represents a group shown by any one of the following groups:



wherein X represents $—CH_2R^1$, $—O—CH_2R^1$, $—S—CH_2R^1$, $—NR_2R^3$ or F;

R^1 represents H or an alkyl;

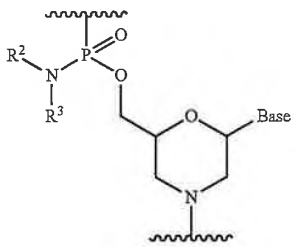
R^2 and R^3, which may be the same or different, each represents H, an alkyl, a cycloalkyl or an aryl;

Y_1 represents O, S, CH_2 or NR^1;

Y_2 represents O, S or NR^1;

Z represents O or S.

Preferably, the morpholino oligomer is an oligomer comprising a constituent unit represented by general formula below (phosphorodiamidate morpholino oligomer (hereinafter referred to as "PMO")).

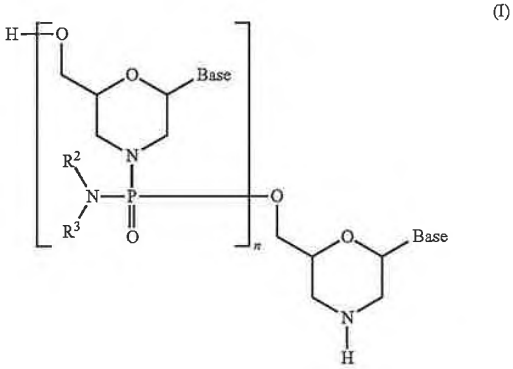


wherein Base, R^2 and R^3 have the same significance as defined above.

The morpholino oligomer may be produced in accordance with, e.g., WO 1991/009033 or WO 2009/064471. In particular, PMO can be produced by the procedure described in WO 2009/064471 or produced by the process shown below.

[Method for Producing PMO]

An embodiment of PMO is, for example, the compound represented by general formula (I) below (hereinafter PMO (I)).



(I)

wherein Base, R^2 and R^3 have the same significance as defined above; and,

n is a given integer of 1 to 99, preferably a given integer of 18 to 28.

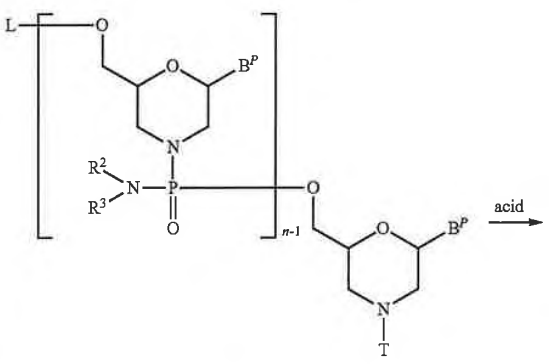
PMO (I) can be produced in accordance with a known method, for example, can be produced by performing the procedures in the following steps.

The compounds and reagents used in the steps below are not particularly limited so long as they are commonly used to prepare PMO.

Also, the following steps can all be carried out by the liquid phase method or the solid phase method (using manuals or commercially available solid phase automated synthesizers). In producing PMO by the solid phase method, it is desired to use automated synthesizers in view of simple operation procedures and accurate synthesis.

(1) Step A:

The compound represented by general formula (II) below (hereinafter referred to as Compound (II)) is reacted with an acid to prepare the compound represented by general formula (III) below (hereinafter referred to as Compound (III)):



(II)

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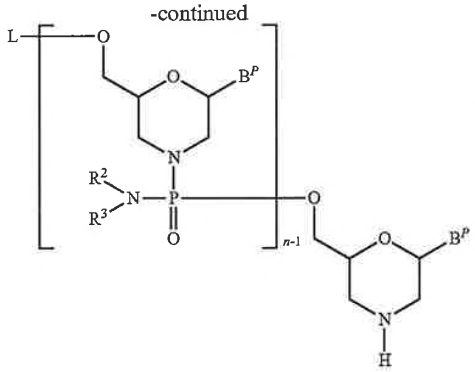


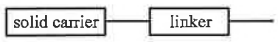
(III)

wherein n, R^2 and R^3 have the same significance as defined above;

each B^P independently represents a nucleobase which may optionally be protected;

T represents trityl, monomethoxytrityl or dimethoxytrityl; and,

L represents hydrogen, an acyl or a group represented by general formula (IV) below (hereinafter referred to as group (IV)).



(IV)

The "nucleobase" for B^P includes the same "nucleobase" as in Base, provided that the amino or hydroxy group in the nucleobase shown by B^P may be protected.

Such protective group for amino is not particularly limited so long as it is used as a protective group for nucleic acids. Specific examples include benzoyl, 4-methoxybenzoyl, acetyl, propionyl, butyryl, isobutyryl, phenylacetyl, phenoxyacetyl, 4-tert-butylphenoxyacetyl, 4-isopropylphenoxyacetyl and (dimethylamino)methylene. Specific examples of the protective group for the hydroxy group include 2-cyanoethyl, 4-nitrophenethyl, phenylsulfonylethyl, methylsulfonylethyl and trimethylsilylethyl, and phenyl, which may be substituted by 1 to 5 electron-withdrawing group at optional substitutable positions, diphenylcarbamoyl, dimethylcarbamoyl, diethylcarbamoyl, methylphenylcarbamoyl, 1-pyrolidinylcarbamoyl, morpholinocarbamoyl, 4-(tert-butylcarboxy) benzyl, 4-[(dimethylamino)carboxy]benzyl and 4-(phenylcarboxy)benzyl, (cf., e.g., WO 2009/064471).

The "solid carrier" is not particularly limited so long as it is a carrier usable for the solid phase reaction of nucleic acids. It is desired for the solid carrier to have the following properties: e.g., (i) it is sparingly soluble in reagents that can be used for the synthesis of morpholino nucleic acid derivatives (e.g., dichloromethane, acetonitrile, tetrazole, N-methylimidazole, pyridine, acetic anhydride, lutidine, trifluoroacetic acid); (ii) it is chemically stable to the reagents usable for the synthesis of morpholino nucleic acid derivatives; (iii) it can be chemically modified; (iv) it can be charged with desired morpholino nucleic acid derivatives; (v) it has a strength sufficient to withstand high pressure through treatments; and (vi) it has a uniform particle diameter range and distribution. Specifically, swellable polystyrene (e.g., aminomethyl polystyrene resin 1% dibenzylbenzene crosslinked (200-400 mesh) (2.4-3.0 mmol/g) (manufactured by Tokyo Chemical Industry), Aminomethylated Polystyrene Resin-.HCl [dibenzylbenzene 1%, 100-200 mesh] (manufactured by Peptide Institute, Inc.)), non-swellable polystyrene (e.g., Primer Support (manufactured by GE Healthcare)), PEG chain-attached polystyrene (e.g., NH_2-PEG resin (manufactured by Watanabe Chemical Co.), TentaGel resin), controlled pore glass (controlled pore glass; CPG) (manufactured by, e.g., CPG), oxalyl-controlled pore glass (cf., e.g., Alul et al., Nucleic Acids Research, Vol. 19, 1527 (1991)), TentaGel support-aminopolyethylene glycol-derivatized support (e.g., Wright et al., cf., Tetrahedron Letters, Vol. 34, 3373 (1993)), and a copolymer of Poros-polystyrene/divinylbenzene.

A "linker" which can be used is a known linker generally used to connect nucleic acids or morpholino nucleic acid derivatives. Examples include 3-aminopropyl, succinyl, 2,2'-diethanolsulfonyl and a long chain alkyl amino (LCAA).

This step can be performed by reacting Compound (11) with an acid.

The "acid" which can be used in this step includes, for example, trifluoroacetic acid, dichloroacetic acid and trichloroacetic acid. The acid used is appropriately in a range of, for example, 0.1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (II), preferably in a range of 1 mol equivalent to 100 mol equivalents based on 1 mol of Compound (II).

An organic amine can be used in combination with the acid described above. The organic amine is not particularly limited and includes, for example, triethylamine. The amount of the organic amine used is appropriately in a range of, e.g., 0.01 mol equivalent to 10 mol equivalents, and preferably in a range of 0.1 mol equivalent to 2 mol equivalents, based on 1 mol of the acid.

When a salt or mixture of the acid and the organic amine is used in this step, the salt or mixture includes, for example, a salt or mixture of trifluoroacetic acid and triethylamine, and more specifically, a mixture of 1 equivalent of triethylamine and 2 equivalents of trifluoroacetic acid.

The acid which can be used in this step may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

The reaction temperature in the reaction described above is preferably in a range of, e.g., 10° C. to 50° C., more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C.

The reaction time may vary depending upon kind of the acid used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

After completion of this step, a base may be added, if necessary, to neutralize the acid remained in the system. The "base" is not particularly limited and includes, for example, diisopropylamine. The base may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% (v/v) to 30% (v/v).

The solvent used in this step is not particularly limited so long as it is inert to the reaction, and includes dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, and a mixture thereof. The reaction temperature is preferably in a range of, e.g., 10° C. to 50° C.,

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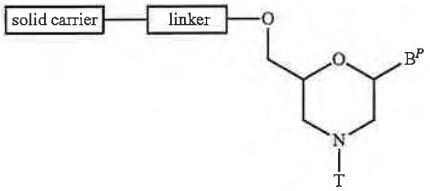
more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C.

The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

In Compound (II), the compound of general formula (IIa) below (hereinafter Compound (IIa)), wherein n is 1 and L is a group (IV), can be produced by the following procedure.

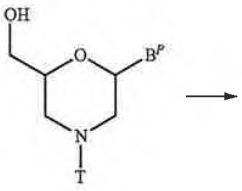
(IIa)



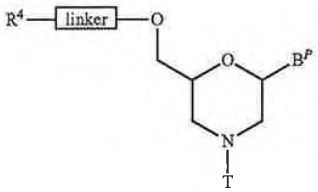
wherein B^P, T, linker and solid carrier have the same significance as defined above.

Step 1:

The compound represented by general formula (V) below is reacted with an acylating agent to prepare the compound represented by general formula (VI) below (hereinafter referred to as Compound (VI)).



(V)



(VI)

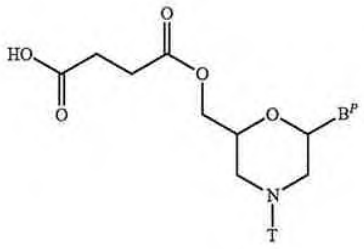
wherein B^P, T and linker have the same significance as defined above; and,

R^4 represents hydroxy, a halogen or amino.

This step can be carried out by known procedures for introducing linkers, using Compound (V) as the starting material.

In particular, the compound represented by general formula (VIa) below can be produced by performing the method known as esterification, using Compound (V) and succinic anhydride.

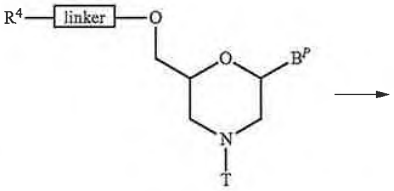
(VIa)



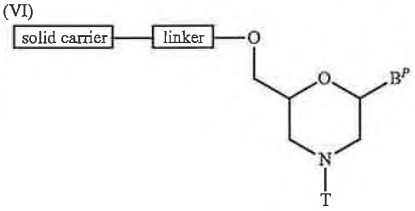
wherein B^P and T have the same significance as defined above.

Step 2:

Compound (VI) is reacted with a solid career by a condensing agent to prepare Compound (IIa).



(VI)



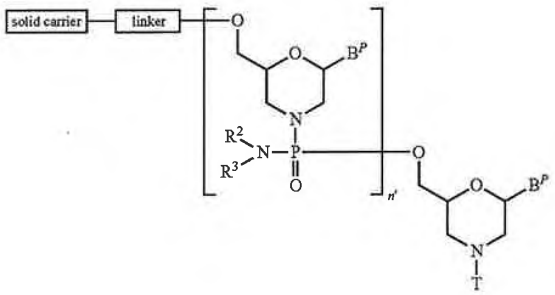
(IIa)

wherein B^P, R^4, T, linker and solid carrier have the same significance as defined above.

This step can be performed using Compound (VI) and a solid carrier in accordance with a process known as condensation reaction.

In Compound (II), the compound represented by general formula (IIa2) below wherein n is 2 to 99 and L is a group represented by general formula (IV) can be produced by using Compound (IIa) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

(IIa2)



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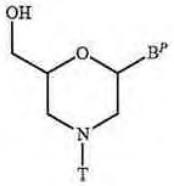
wherein B^P, R^2, R^3, T, linker and solid carrier have the same significance as defined above; and,
n' represents 1 to 98.

In Compound (II), the compound of general formula (IIb) below wherein n is 1 and L is hydrogen can be produced by the procedure described in, e.g., WO 1991/009033.

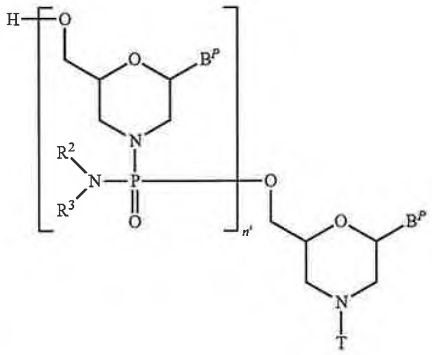
(IIb)



wherein B^P and T have the same significance as defined above.

In Compound (II), the compound represented by general formula (IIb2) below wherein n is 2 to 99 and L is hydrogen can be produced by using Compound (IIb) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

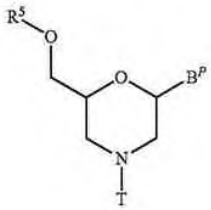
(IIb2)



wherein B^P, n', R^2, R^3 and T have the same significance as defined above.

In Compound (II), the compound represented by general formula (IIc) below wherein n is 1 and L is an acyl can be produced by performing the procedure known as acylation reaction, using Compound (IIb).

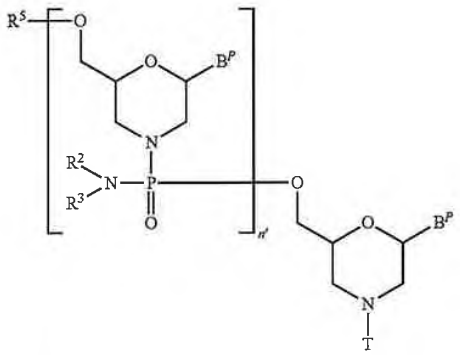
(IIc)



wherein B^P and T have the same significance as defined above; and,
R^5 represents an acyl.

In Compound (II), the compound represented by general formula (IIc2) below wherein n is 2 to 99 and L is an acyl can be produced by using Compound (IIc) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

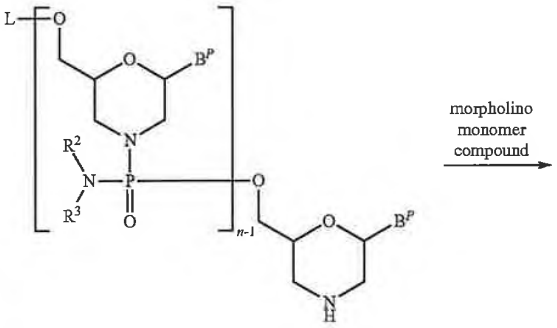
(IIc2)



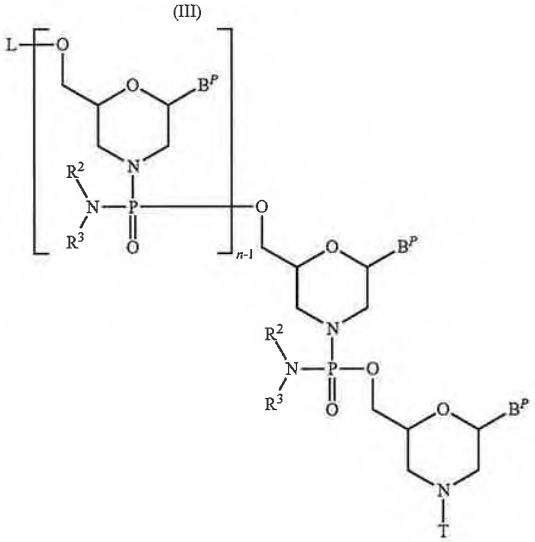
wherein B^P, n', R^2, R^3, R^5 and T have the same significance as defined above.

(2) Step B

Compound (III) is reacted with a morpholino monomer compound in the presence of a base to prepare the compound represented by general formula (VII) below (hereinafter referred to as Compound (VII)):



(III)



(VII)

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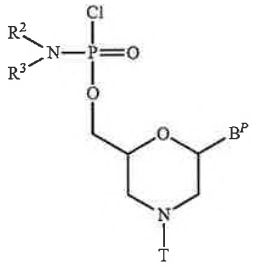
wherein B^P, L, n, R^2, R^3 and T have the same significance as defined above.

This step can be performed by reacting Compound (III) with the morpholino monomer compound in the presence of a base.

The morpholino monomer compound includes, for example, compounds represented by general formula (VIII) below:

(VIII)



wherein B^P, R^2, R^3 and T have the same significance as defined above.

The "base" which can be used in this step includes, for example, diisopropylamine, triethylamine and N-ethylmorpholine. The amount of the base used is appropriately in a range of 1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (III), preferably, 10 mol equivalents to 100 mol equivalents based on 1 mol of Compound (III).

The morpholino monomer compound and base which can be used in this step may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, N,N-dimethylimidazolidone, N-methylpiperidone, DMF, dichloromethane, acetonitrile, tetrahydrofuran, or a mixture thereof.

The reaction temperature is preferably in a range of, e.g., 0° C. to 100° C., and more preferably, in a range of 10° C. to 50° C.

The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 1 minute to 48 hours in general, and preferably in a range of 30 minutes to 24 hours.

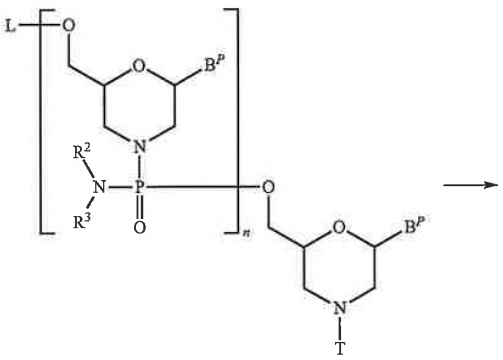
Furthermore, after completion of this step, an acylating agent can be added, if necessary. The "acylating agent" includes, for example, acetic anhydride, acetyl chloride and phenoxyacetic anhydride. The acylating agent may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol(s) (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

If necessary, a base such as pyridine, lutidine, collidine, triethylamine, diisopropylethylamine, N-ethylmorpholine, etc. may also be used in combination with the acylating agent. The amount of the acylating agent is appropriately in a range of 0.1 mol equivalent to 10000 mol equivalents, and preferably in a range of 1 mol equivalent to 1000 mol equivalents. The amount of the base is appropriately in a range of, e.g., 0.1 mol equivalent to 100 mol equivalents, and preferably in a range of 1 mol equivalent to 10 mol equivalents, based on 1 mol of the acylating agent.

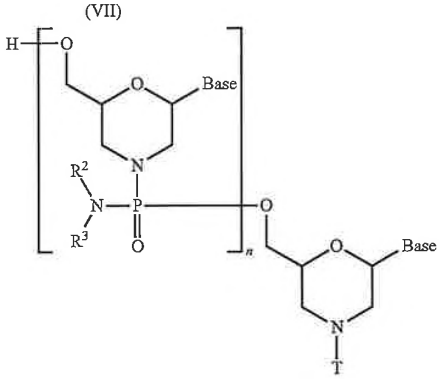
The reaction temperature in this reaction is preferably in a range of 10° C. to 50° C., more preferably, in a range of 10° C. to 50° C., much more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C. The reaction time may vary depending upon kind of the acylating agent used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

(3) Step C:

In Compound (VII) produced in Step B, the protective group is removed using a deprotecting agent to prepare the compound represented by general formula (IX).



(VII)



(IX)

wherein Base, B^P, L, n, R^2, R^3 and T have the same significance as defined above.

This step can be performed by reacting Compound (VII) with a deprotecting agent.

The "deprotecting agent" includes, e.g., conc. ammonia water and methylamine. The "deprotecting agent" used in this step may also be used as a dilution with, e.g., water, methanol, ethanol, isopropyl alcohol, acetonitrile, tetrahydrofuran, DMF, N,N-dimethylimidazolidone, N-methylpiperidone, or a mixture of these solvents. Among others, ethanol is preferred. The amount of the deprotecting agent used is appropriately in a range of, e.g., 1 mol equivalent to 100000 mol equivalents, and preferably in a range of 10 mol equivalents to 1000 mol equivalents, based on 1 mol of Compound (VII).

The reaction temperature is appropriately in a range of 15° C. to 75° C., preferably, in a range of 40° C. to 70° C., and more preferably, in a range of 50° C. to 60° C. The reaction time for deprotection may vary depending upon kind of Compound (VII), reaction temperature, etc., and is appropriately in a range of 10 minutes to 30 hours, preferably 30 minutes to 24 hours, and more preferably in a range of 5 hours to 20 hours.

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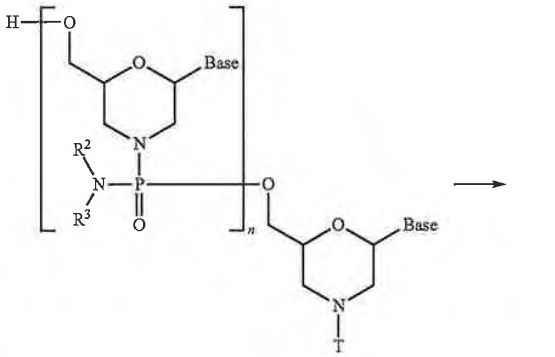
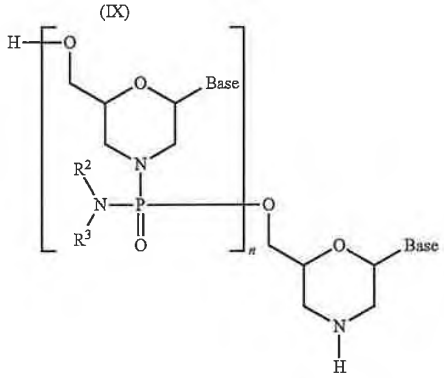
(4) Step D:

PMO (I) is produced by reacting Compound (IX) produced in step C with an acid:



(IX)



(I)

wherein Base, n, R^2, R^3 and T have the same significance as defined above.

This step can be performed by adding an acid to Compound (IX).

The "acid" which can be used in this step includes, for example, trichloroacetic acid, dichloroacetic acid, acetic acid, phosphoric acid, hydrochloric acid, etc. The acid used is appropriately used to allow the solution to have a pH range of 0.1 to 4.0, and more preferably, in a range of pH 1.0 to 3.0. The solvent is not particularly limited so long as it is inert to the reaction, and includes, for example, acetonitrile, water, or a mixture of these solvents thereof.

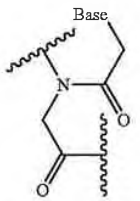
The reaction temperature is appropriately in a range of 10° C. to 50° C., preferably, in a range of 20° C. to 40° C., and more preferably, in a range of 25° C. to 35° C. The reaction time for deprotection may vary depending upon kind of Compound (IX), reaction temperature, etc., and is appropriately in a range of 0.1 minute to 5 hours, preferably 1 minute to 1 hour, and more preferably in a range of 1 minute to 30 minutes.

PMO (I) can be obtained by subjecting the reaction mixture obtained in this step to conventional means of separation and purification such as extraction, concentration, neutralization, filtration, centrifugal separation, recrystallization, reversed phase column chromatography C_8 to C_{18}, cation exchange column chromatography, anion exchange column chromatography, gel filtration column chromatography, high performance liquid chromatography, dialysis, ultrafiltration, etc., alone or in combination thereof. Thus, the desired PMO (I) can be isolated and purified (cf., e.g., WO 1991/09033).

In purification of PMO (I) using reversed phase chromatography, e.g., a solution mixture of 20 mM triethylamine/acetate buffer and acetonitrile can be used as an elution solvent.

In purification of PMO (I) using ion exchange chromatography, e.g., a solution mixture of 1 M saline solution and 10 mM sodium hydroxide aqueous solution can be used as an elution solvent.

A peptide nucleic acid is the oligomer of the present invention having a group represented by the following general formula as the constituent unit:



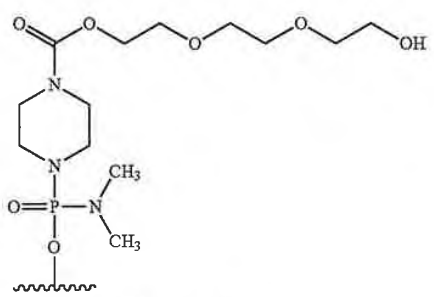
wherein Base has the same significance as defined above.

Peptide nucleic acids can be prepared by referring to, e.g., the following literatures.

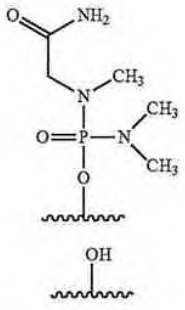
1) P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, Science, 254, 1497 (1991)
2) M. Egholm, O. Buchardt, P. E. Nielsen, R. H. Berg, Jacs., 114, 1895 (1992)
3) K. L. Dueholm, M. Egholm, C. Behrens, L. Christensen, H. F. Hansen, T. Vulpius, K. H. Petersen, R. H. Berg, P. E. Nielsen, O. Buchardt, J. Org. Chem., 59, 5767 (1994)
4) L. Christensen, R. Fitzpatrick, B. Gildea, K. H. Petersen, H. E Hansen, T. Koch, M. Egholm, O. Buchardt, P. E. Nielsen, J. Coull, R. H. Berg, J. Pept. Sci., 1, 175 (1995)
5) T. Koch, H. F. Hansen, P. Andersen, T. Larsen, H. G Batz, K. Otteson, H. Orum, J. Pept. Res., 49, 80 (1997)

In the oligomer of the present invention, the 5' end may be any of chemical structures (1) to (3) below, and preferably is (3)-OH.

(1)



(2)



(3)

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Hereinafter, the groups shown by (1), (2) and (3) above are referred to as "Group (1)," "Group (2)" and "Group (3)," respectively.

2. Pharmaceutical Composition

The oligomer of the present invention causes exon 53 skipping with a higher efficiency as compared to the prior art antisense oligomers. It is thus expected that conditions of muscular dystrophy can be relieved with high efficience by administering the pharmaceutical composition comprising the oligomer of the present invention to DMD patients. For example, when the pharmaceutical composition comprising the oligomer of the present invention is used, the same therapeutic effects can be achieved even in a smaller dose than that of the oligomers of the prior art. Accordingly, side effects can be alleviated and such is economical.

In another embodiment, the present invention provides the pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the oligomer of the present invention, a pharmaceutically acceptable salt or hydrate thereof (hereinafter referred to as "the composition of the present invention").

Examples of the pharmaceutically acceptable salt of the oligomer of the present invention contained in the composition of the present invention are alkali metal salts such as salts of sodium, potassium and lithium; alkaline earth metal salts such as salts of calcium and magnesium; metal salts such as salts of aluminum, iron, zinc, copper, nickel, cobalt, etc.; ammonium salts; organic amine salts such as salts of t-octylamine, dibenzylamine, morpholine, glucosamine, phenylglycine alkyl ester, ethylenediamine, N-methylglucamine, guanidine, diethylamine, triethylamine, dicyclohexylamine, N,N'-dibenzylethylenediamine, chloroprocaine, procaine, diethanolamine, N-benzylphenethylamine, piperazine, tetramethylammonium, tris(hydroxymethyl)aminomethane; hydrohalide salts such as salts of hydrofluorates, hydrochlorides, hydrobromides and hydroiodides; inorganic acid salts such as nitrates, perchlorates, sulfates, phosphates, etc.; lower alkane sulfonates such as methanesulfonates, trifluoromethanesulfonates and ethanesulfonates; arylsulfonates such as benzenesulfonates and p-toluenesulfonates; organic acid salts such as acetates, malates, fumarates, succinates, citrates, tartarates, oxalates, maleates, etc.; and, amino acid salts such as salts of glycine, lysine, arginine, ornithine, glutamic acid and aspartic acid. These salts may be produced by known methods. Alternatively, the oligomer of the present invention contained in the composition of the present invention may be in the form of a hydrate thereof.

Administration route for the composition of the present invention is not particularly limited so long as it is pharmaceutically acceptable route for administration, and can be chosen depending upon method of treatment. In view of easiness in delivery to muscle tissues, preferred are intravenous administration, intraarterial administration, intramuscular administration, subcutaneous administration, oral administration, tissue administration, transdermal administration, etc. Also, dosage forms which are available for the composition of the present invention are not particularly limited, and include, for example, various injections, oral agents, drips, inhalations, ointments, lotions, etc.

In administration of the oligomer of the present invention to patients with muscular dystrophy, the composition of the present invention preferably contains a carrier to promote delivery of the oligomer to muscle tissues. Such a carrier is not particularly limited as far as it is pharmaceutically acceptable, and examples include cationic carriers such as cationic liposomes, cationic polymers, etc., or carriers using viral envelope. The cationic liposomes are, for example, liposomes composed of 2-O-(2-diethylaminoethyl)carabamoyl-1,3-O-dioleoylglycerol and phospholipids as the essential constituents (hereinafter referred to as "liposome A"), Oligofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectin (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine 2000 (registered trademark) (manufactured by Invitrogen Corp.), DMRIE-C (registered trademark) (manufactured by Invitrogen Corp.), GeneSilencer (registered trademark) (manufactured by Gene Therapy Systems), TransMessenger (registered trademark) (manufactured by QIAGEN, Inc.), TransIT TKO (registered trademark) (manufactured by Mirus) and Nucleofector II (Lonza). Among others, liposome A is preferred. Examples of cationic polymers are JetSI (registered trademark) (manufactured by Qbiogene, Inc.) and Jet-PEI (registered trademark) (polyethylenimine, manufactured by Qbiogene, Inc.). An example of carriers using viral envelop is GenomeOne (registered trademark) (HVJ-E liposome, manufactured by Ishihara Sangyo). Alternatively, the medical devices described in Japanese Patent No. 2924179 and the cationic carriers described in Japanese Domestic Re-Publication PCT Nos. 2006/129594 and 2008/096690 may be used as well.

A concentration of the oligomer of the present invention contained in the composition of the present invention may vary depending on kind of the carrier, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the oligomer of the present invention contained in the composition of the present invention and the carrier (carrier/oligomer of the present invention) may vary depending on property of the oligomer, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20.

In addition to the oligomer of the present invention and the carrier described above, pharmaceutically acceptable additives may also be optionally formulated in the composition of the present invention. Examples of such additives are emulsification aids (e.g., fatty acids having 6 to 22 carbon atoms and their pharmaceutically acceptable salts, albumin and dextran), stabilizers (e.g., cholesterol and phosphatidic acid), isotonizing agents (e.g., sodium chloride, glucose, maltose, lactose, sucrose, trehalose), and pH controlling agents (e.g., hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid, sodium hydroxide, potassium hydroxide and triethanolamine). One or more of these additives can be used. The content of the additive in the composition of the present invention is appropriately 90 wt % or less, preferably 70 wt % or less and more preferably, 50 wt % or less.

The composition of the present invention can be prepared by adding the oligomer of the present invention to a carrier dispersion and adequately stirring the mixture. Additives may be added at an appropriate step either before or after addition of the oligomer of the present invention. An aqueous solvent that can be used in adding the oligomer of the present invention is not particularly limited as far as it is pharmaceutically acceptable, and examples are injectable water or injectable distilled water, electrolyte fluid such as physiological saline, etc., and sugar fluid such as glucose fluid, maltose fluid, etc. A person skilled in the art can appropriately choose conditions for pH and temperature for such matter.

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The composition of the present invention may be prepared into, e.g., a liquid form and its lyophilized preparation. The lyophilized preparation can be prepared by lyophilizing the composition of the present invention in a liquid form in a conventional manner. The lyophilization can be performed, for example, by appropriately sterilizing the composition of the present invention in a liquid form, dispensing an aliquot into a vial container, performing preliminary freezing for 2 hours at conditions of about −40 to −20° C., performing a primary drying at 0 to 10° C. under reduced pressure, and then performing a secondary drying at about 15 to 25° C. under reduced pressure. In general, the lyophilized preparation of the composition of the present invention can be obtained by replacing the content of the vial with nitrogen gas and capping.

The lyophilized preparation of the composition of the present invention can be used in general upon reconstitution by adding an optional suitable solution (reconstitution liquid) and redissolving the preparation. Such a reconstitution liquid includes injectable water, physiological saline and other infusion fluids. A volume of the reconstitution liquid may vary depending on the intended use, etc., is not particularly limited, and is suitably 0.5 to 2-fold greater than the volume prior to lyophilization or no more than 500 mL.

It is desired to control a dose of the composition of the present invention to be administered, by taking the following factors into account: the type and dosage form of the oligomer of the present invention contained; patients' conditions including age, body weight, etc.; administration route; and the characteristics and extent of the disease. A daily dose calculated as the amount of the oligomer of the present invention is generally in a range of 0.1 mg to 10 g/human, and preferably 1 mg to 1 g/human. This numerical range may vary occasionally depending on type of the target disease, administration route and target molecule. Therefore, a dose lower than the range may be sufficient in some occasion and conversely, a dose higher than the range may be required occasionally. The composition can be administered from once to several times daily or at intervals from one day to several days.

In still another embodiment of the composition of the present invention, there is provided a pharmaceutical composition comprising a vector capable of expressing the oligonucleotide of the present invention and the carrier described above. Such an expression vector may be a vector capable of expressing a plurality of the oligonucleotides of the present invention. The composition may be formulated with pharmaceutically acceptable additives as in the case with the composition of the present invention containing the oligomer of the present invention. A concentration of the expression vector contained in the composition may vary depending upon type of the career, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the expression vector contained in the composition and the carrier (carrier/expression vector) may vary depending on property of the expression vector, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20. The content of the carrier contained in the composition is the same as in the case with the composition of the present invention containing the oligomer of the present invention, and a method for producing the same is also the same as in the case with the composition of the present invention.

Hereinafter, the present invention will be described in more detail with reference to EXAMPLES and TEST EXAMPLES below, but is not deemed to be limited thereto.

EXAMPLES

Reference Example 1

4-{[(2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic Acid Loaded onto Aminomethyl Polystyrene Resin

Step 1: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic Acid

Under argon atmosphere, 22.0 g of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide and 7.04 g of 4-dimethylaminopyridine (4-DMAP) were suspended in 269 mL of dichloromethane, and 5.76 g of succinic anhydride was added to the suspension, followed by stirring at room temperature for 3 hours. To the reaction solution was added 40 mL of methanol, and the mixture was concentrated under reduced pressure. The residue was extracted using ethyl acetate and 0.5M aqueous potassium dihydrogenphosphate solution. The resulting organic layer was washed sequentially with 0.5M aqueous potassium dihydrogenphosphate solution, water and brine in the order mentioned. The resulting organic layer was dried over sodium sulfate and concentrated under reduced pressure to give 25.9 g of the product.

Step 2: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic Acid Loaded onto Aminomethyl Polystyrene Resin

After 23.5 g of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid was dissolved in 336 mL of pyridine (dehydrated), 4.28 g of 4-DMAP and 40.3 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were added to the solution. Then, 25.0 g of Aminomethyl Polystyrene Resin cross-linked with 1% DVB (manufactured by Tokyo Chemical Industry Co., Ltd., A1543) and 24 mL of triethylamine were added to the mixture, followed by shaking at room temperature for 4 days. After completion of the reaction, the resin was taken out by filtration. The resulting resin was washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure. To the resulting resin were added 150 mL of tetrahydrofuran (dehydrate), 15 mL of acetic anhydride and 15 mL of 2,6-lutidine, and the mixture was shaken at room temperature for 2 hours. The resin was taken out by filtration, washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure to give 33.7 g of the product.

The loading amount of the product was determined by measuring UV absorbance at 409 nm of the molar amount of the trityl per g resin using a known method. The loading amount of the resin was 397.4 μmol/g.

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Conditions of UV Measurement
Device: U-2910 (Hitachi, Ltd.)
Solvent: methanesulfonic acid
Wavelength: 265 nm
ε Value: 45000

Reference Example 2

4-Oxo-4-{[(2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}butanoic acid Loaded onto 2-aminomethylpolystyrene Resin

Step 1: Production of N^2-(phenoxyacetyl)guanosine

Guanosine, 100 g, was dried at 80° C. under reduced pressure for 24 hours. After 500 mL of pyridine (anhydrous) and 500 mL of dichloromethane (anhydrous) were added thereto, 401 mL of chlorotrimethylsilane was dropwise added to the mixture under an argon atmosphere at 0° C., followed by stirring at room temperature for 3 hours. The mixture was again ice-cooled and 66.3 g of phenoxyacetyl chloride was dropwise added thereto. Under ice cooling, the mixture was stirred for further 3 hours. To the reaction solution was added 500 mL of methanol, and the mixture was stirred at room temperature overnight. The solvent was then removed by distillation under reduced pressure. To the residue was added 500 mL of methanol, and concentration under reduced pressure was performed 3 times. To the residue was added 4 L of water, and the mixture was stirred for an hour under ice cooling. The precipitates formed were taken out by filtration, washed sequentially with water and cold methanol and then dried to give 150.2 g of the objective compound (yield: 102%) (cf. Org. Lett. (2004), Vol. 6, No. 15, 2555-2557).

Step 2: N-{9-[(2R,6S)-6-(hydroxymethyl)-4-morpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide p-toluenesulfonate

In 480 mL of methanol was suspended 30 g of the compound obtained in Step 1, and 130 mL of 2N hydrochloric acid was added to the suspension under ice cooling. Subsequently, 56.8 g of ammonium tetraborate tetrahydrate and 16.2 g of sodium periodate were added to the mixture in the order mentioned and stirred at room temperature for 3 hours. The reaction solution was ice cooled and the insoluble matters were removed by filtration, followed by washing with 100 mL of methanol. The filtrate and washing liquid were combined and the mixture was ice cooled. To the mixture was added 11.52 g of 2-picoline borane. After stirring for 20 minutes, 54.6 g of p-toluenesulfonic acid monohydrate was slowly added to the mixture, followed by stirring at 4° C. overnight. The precipitates were taken out by filtration and washed with 500 mL of cold methanol and dried to give 17.7 g of the objective compound (yield: 43.3%).

^{1}H NMR (δ, DMSO-d6): 9.9-9.2 (2H, br), 8.35 (1H, s), 7.55 (2H, m), 7.35 (2H, m), 7.10 (2H, d, J=7.82 Hz), 7.00 (3H, m), 5.95 (1H, dd, J=10.64, 2.42 Hz), 4.85 (2H, s), 4.00 (1H, m), 3.90-3.60 (2H, m), 3.50-3.20 (5H, m), 2.90 (1H, m), 2.25 (3H, s)

Step 3: Production of N-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide

In 30 mL of dichloromethane was suspended 2.0 g of the compound obtained in Step 2, and 13.9 g of triethylamine and 18.3 g of trityl chloride were added to the suspension under ice cooling. The mixture was stirred at room temperature for an hour. The reaction solution was washed with saturated sodium bicarbonate aqueous solution and then with water, and dried. The organic layer was concentrated under reduced pressure. To the residue was added 40 mL of 0.2M sodium citrate buffer (pH 3)/methanol (1:4 (v/v)), and the mixture was stirred. Subsequently, 40 mL of water was added and the mixture was stirred for an hour under ice cooling. The mixture was taken out by filtration, washed with cold methanol and dried to give 1.84 g of the objective compound (yield: 82.0%).

Step 4: Production of 4-oxo-4-{[(2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}butanoic Acid Loaded onto Aminomethyl Polystyrene Resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that N-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

Reference Example 3

4-{[(2S,6R)-6-(5-Methyl-2,4-dioxo-3,4-dihydropyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic Acid Loaded onto Aminomethyl Polystyrene Resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-5-methylpyrimidine-2,4(1H,3H)-dione was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

Reference Example 4

1,12-Dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic Acid Loaded onto Aminomethyl Polystyrene Resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 2-[2-(2-hydroxyethoxy)ethoxy]ethyl 4-tritylpiperazine-1-carboxylic acid (the compound described in WO 2009/064471) was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide.

According to the descriptions in EXAMPLES 1 to 12 and REFERENCE EXAMPLES 1 to 3 below, various types of PMO shown by PMO Nos. 1-11 and 13-16 in TABLE 2 were synthesized. The PMO synthesized was dissolved in injectable water (manufactured by Otsuka Pharmaceutical Factory, Inc.). PMO No. 12 was purchased from Gene Tools, LLC.

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TABLE 2

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
1	31-55	5' end: group (3)	SEQ ID NO: 4
2	32-53	5' end: group (3)	SEQ ID NO: 8
3	32-56	5' end: group (3)	SEQ ID NO: 11
4	33-54	5' end: group (3)	SEQ ID NO: 15
5	34-58	5' end: group (3)	SEQ ID NO: 25
6	36-53	5' end: group (3)	SEQ ID NO: 32
7	36-55	5' end: group (3)	SEQ ID NO: 34
8	36-56	5' end: group (3)	SEQ ID NO: 35
9	36-57	5' end: group (3)	SEQ ID NO: 36
10	33-57	5' end: group (3)	SEQ ID NO: 18
11	39-69	Sequence corresponding to H53A(+39 + 69) (cf. Table in 1) Non-Patent Document 3, 5' end: group (3)	SEQ ID NO: 38
12	30-59	Sequence corresponding to h53A30/1 (cf. Table 1) in Non-Patent Document 5, 5' end: group (2)	SEQ ID NO: 39
13	32-56	5' end: group (1)	SEQ ID NO: 11
14	36-56	5' end: group (1)	SEQ ID NO: 35
15	30-59	Sequence corresponding to h53A30/1 (cf. Table 1) in Non-Patent Document 5 5' end: group (3)	SEQ ID NO: 39
16	23-47	Sequence corresponding to SEQ ID NO: 429 described in Patent Document 4, 5' end: group (3)	SEQ ID NO: 47

Example 1

PMO No. 8

4-{[(2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid, loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 1), 2 g (800 μmop was transferred to a reaction vessel, and 30 mL of dichloromethane was added thereto. The mixture was allowed to stand for 30 minutes. After the mixture was further washed twice with 30 mL of dichloromethane, the following synthesis cycle was started. The desired morpholino monomer compound was added in each cycle to give the nucleotide sequence of the title compound.

TABLE 3

Step	Reagent	Volume (mL)	Time (mm)
1	deblocking solution	30	2.0
2	deblocking solution	30	2.0
3	deblocking solution	30	2.0
4	deblocking solution	30	2.0
5	deblocking solution	30	2.0
6	deblocking solution	30	2.0
7	neutralizing solution	30	1.5
8	neutralizing solution	30	1.5
9	neutralizing solution	30	1.5
10	neutralizing solution	30	1.5
11	neutralizing solution	30	1.5
12	neutralizing solution	30	1.5
13	dichloromethane	30	0.5
14	dichloromethane	30	0.5
15	dichloromethane	30	0.5
16	coupling solution B	20	0.5
17	coupling solution A	6-11	90.0
18	dichloromethane	30	0.5
19	dichloromethane	30	0.5
20	dichloromethane	30	0.5
21	capping solution	30	3.0

TABLE 3-continued

Step	Reagent	Volume (mL)	Time (mm)
22	capping solution	30	3.0
23	dichloromethane	30	0.5
24	dichloromethane	30	0.5
25	dichloromethane	30	0.5

The deblocking solution used was a solution obtained by dissolving a mixture of trifluoroacetic acid (2 equivalents) and triethylamine (1 equivalent) in a dichloromethane solution containing 1% (v/v) ethanol and 10% (v/v) 2,2,2-trifluoroethanol to be 3% (w/v). The neutralizing solution used was a solution obtained by dissolving N,N-diisopropylethylamine in a dichloromethane solution containing 25% (v/v) 2-propanol to be 5% (v/v). The coupling solution A used was a solution obtained by dissolving the morpholino monomer compound in 1,3-dimethyl-2-imidazolidinone containing 10% (v/v) N,N-diisopropylethylamine to be 0.15M. The coupling solution B used was a solution obtained by dissolving N,N-diisopropylethylamine in 1,3-dimethyl-2-imidazolidinone to be 10% (v/v). The capping solution used was a solution obtained by dissolving 20% (v/v) acetic anhydride and 30% (v/v) 2,6-lutidine in dichloromethane.

The aminomethyl polystyrene resin loaded with the PMO synthesized above was recovered from the reaction vessel and dried at room temperature for at least 2 hours under reduced pressure. The dried PMO loaded onto aminomethyl polystyrene resin was charged in a reaction vessel, and 200 mL of 28% ammonia water-ethanol (1/4) was added thereto. The mixture was stirred at 55° C. for 15 hours. The aminomethyl polystyrene resin was separated by filtration and washed with 50 mL of water-ethanol (1/4). The resulting filtrate was concentrated under reduced pressure. The resulting residue was dissolved in 100 mL of a solvent mixture of 20 mM acetic acid-triethylamine buffer (TEAA buffer) and acetonitrile (4/1) and filtered through a membrane filter. The filtrate obtained was purified by reversed phase HPLC. The conditions used are as follows.

TABLE 4

Column	XTerra MS18 (Waters, φ50x 100 mm, 1CV = 200 mL)
Flow rate	60 mL/min
Column temperature	room temperature
Solution A	20 mM TEAA buffer
Solution B	CH_3CN
Gradient	(B) conc. 20→50%/9CV

Each fraction was analyzed and the product was recovered in 100 mL of acetonitrile-water (1/1), to which 200 mL of ethanol was added. The mixture was concentrated under reduced pressure. Further drying under reduced pressure gave a white solid. To the resulting solid was added 300 mL of 10 mM phosphoric acid aqueous solution to suspend the solid. To the suspension was added 10 mL of 2M phosphoric acid aqueous solution, and the mixture was stirred for 15 minutes. Furthermore, 15 mL of 2M sodium hydrate aqueous solution was added for neutralization. Then, 15 mL of 2M sodium hydroxide aqueous solution was added to make the mixture alkaline, followed by filtration through a membrane filter (0.45 μm). The mixture was thoroughly washed with 100 mL of 10 mM sodium hydroxide aqueous solution to give the product as an aqueous solution.

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The resulting aqueous solution containing the product was purified by an anionic exchange resin column. The conditions used are as follows.

TABLE 5

Column	Source 30Q (GE Healthcare, φ40x 150 mm, 1CV = 200 mL)
Flow rate	80 mL/min
Column temp.	room temperature
Solution A	10 mM sodium hydroxide aqueous solution
Solution B	10 mM sodium hydroxide aqueous solution, 1M sodium chloride aqueous solution
Gradient	(B) conc. 5→35%/15CV

Each fraction was analyzed (on HPLC) and the product was obtained as an aqueous solution. To the resulting aqueous solution was added 225 mL of 0.1M phosphate buffer (pH 6.0) for neutralization. The mixture was filtered through a membrane filter (0.45 μm). Next, ultrafiltration was performed under the conditions described below.

TABLE 6

Filter	PELLICON2 MINI FILTER PLBC 3K Regenerated Cellulose, Screen Type C
Size	0.1 m^2

The filtrate was concentrated to give approximately 250 mL of an aqueous solution. The resulting aqueous solution was filtered through a membrane filter (0.45 μm). The aqueous solution obtained was freeze-dried to give 1.5 g of the objective compound as a white cotton-like solid.

ESI-TOF-MS Calcd.: 6924.82.
Found: 6923.54.

Example 2

PMO. No. 1

The title compound was produced in accordance with the procedure of EXAMPLE 1.

MALDI-TOF-MS Calcd.: 8291.96.
Found: 8296.24.

Example 3

PMO. No. 2

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 7310.13.
Found: 7309.23.

Example 4

PMO. No. 3

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8270.94.
Found: 8270.55.

Example 5

PMO. No. 4

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-trityl-morpholin-2-yl)methoxy)-4-oxobutanoic acid (REFERENCE EXAMPLE 3) loaded onto aminomethyl polystyrene resin was used as the starting material.

ESI-TOF-MS Calcd.: 7310.13.
Found: 7310.17.

Example 6

PMO. No. 5

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-trityl-morpholin-2-yl)methoxy)-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 3) was used as the starting material.

ESI-TOF-MS Calcd.: 8270.94.
Found: 8270.20.

Example 7

PMO. No. 6

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 5964.01.
Found: 5963.68.

Example 8

PMO. No. 7

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 6609.55.
Found: 6608.85.

Example 9

PMO. No. 9

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9(6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 7280.11.
Found: 7279.42.

Example 10

PMO. No. 10

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9(6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 8295.95.
Found: 8295.91.

Example 11

PMO. No. 13

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 7276.15.
Found: 7276.69.

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Example 12

PMO. No. 14

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 8622.27.

Found: 8622.29.

Comparative Example 1

PMO. No. 11

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 10274.63.

Found: 10273.71.

Comparative Example 2

PMO. No. 15

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 9941.33.

Found: 9940.77.

Comparative Example 3

PMO. No. 16

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8238.94.

Found: 8238.69.

Test Example 1

In Vitro Assay

Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 10 μM of the oligomers PMO Nos. 1 to 8 of the present invention and the antisense oligomer PMO No. 11 were transfected with 4×10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

After transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen) under conditions of 37° C. and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

94° C., 2 mins: thermal denaturation

[94° C., 10 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer: 5'-AGGATTTGGAACAGAGGCGTC-3' (SEQ ID NO: 40)

Reverse primer: 5'-GTCTGCCACTGGCGGAGGTC-3' (SEQ ID NO: 41)

Next, a nested PCR was performed with the product amplified by RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94° C., 2 mins: thermal denaturation

[94° C., 15 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3' (SEQ ID NO: 42)

Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3' (SEQ ID NO: 43)

The reaction product, 1 μl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A+B)\times 100$$

Experimental Results

The results are shown in FIG. 1. This experiment revealed that the oligomers PMO Nos. 1 to 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomer PMO No. 11. In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than four times higher exon skipping efficiency than that of the antisense oligomer PMO No. 11.

Test Example 2

In Vitro Assay Using Human Fibroblasts

Human myoD gene (SEQ ID NO: 44) was introduced into TIG-119 cells (human normal tissue-derived fibroblasts, National Institute of Biomedical Innovation) or 5017 cells (human DMD patient-derived fibroblasts, Coriell Institute for Medical Research) using a ZsGreen1 coexpression retroviral vector.

After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at $5\times10^4/cm^2$ into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM.F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

The medium was replaced 24 hours later by differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12 to 14 days to differentiate into myotubes.

Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and the morpholino oligomer was added

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thereto in a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 1 mins; 60° C., 1 mins; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 7 mins: final extension

The primers used were hEX51F and hEX55R.

hEX51F: 5'-CGGGCTTGGACAGAACTTAC-3' (SEQ ID NO: 45)

hEx55R: 5'-TCCTTACGGGTAGCATCCTG-3' (SEQ ID NO: 46)

The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

$$\text{Skipping efficiency (\%)} = A/(A+B)\times 100$$

Experimental Results

The results are shown in FIGS. **2** and **3**. This experiment revealed that in TIG-119 cells, the oligomers PMO Nos. 3, 8 and 9 of the present invention (FIG. **2**) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. **2**). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than twice higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. **2**).

Furthermore, this experiment revealed that the oligomers PMO Nos. 3 and 8 to 10 of the present invention (FIG. **3**) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. **3**). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than seven times higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. **3**).

Test Example 3

In Vitro Assay Using Human Fibroblasts

The skin fibroblast cell line (fibroblasts from human DMD patient (exons 45-52 or exons 48-52)) was established by biopsy from the medial left upper arm of DMD patient with deletion of exons 45-52 or DMD patient with deletion of exons 48-52. Human myoD gene (SEQ ID NO: 44) was introduced into the fibroblast cells using a ZsGreen1 coexpression retroviral vector.

After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at $5\times10^4/cm^2$ into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

The medium was replaced 24 hours later by a differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12, 14 or 20 days to differentiate into myotubes.

Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and a morpholino oligomer was added thereto at a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 1 mins; 60° C., 1 mins; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 7 mins: final extension

The primers used were hEx44F and h55R.

hEx44F: 5'-TGTTGAGAAATGGCGGCGT-3' (SEQ ID NO: 48)

hEx55R: 5'-TCCTTACGGGTAGCATCCTG-3' (SEQ ID NO: 46)

The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

$$\text{Skipping efficiency (\%)} = A/(A+B)\times 100$$

Experimental Results

The results are shown in FIGS. **4** and **5**. This experiment revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with an efficiency as high as more than 80% in the cells from DMD patient with deletion of exons 45-52 (FIG. **4**) or deletion of exons 48-52 (FIG. **5**). Also, the oligomers PMO Nos. 3 and 8 of the present invention were found to cause exon 53 skipping with a higher efficiency than that of the antisense oligomer PMO No. 15 in the cells from DMD patient with deletion of exons 45-52 (FIG. **4**).

Test Example 4

Western Blotting

The oligomer PMO No. 8 of the present invention was added to the cells at a concentration of 10 μM, and proteins were extracted from the cells after 72 hours using a RIPA buffer (manufactured by Thermo Fisher Scientific) containing Complete Mini (manufactured by Roche Applied Science) and quantified using a BCA protein assay kit (manufactured by Thermo Fisher Scientific). The proteins were electrophoresed in NuPAGE Novex Tris-Acetate Gel 3-8% (manufactured by Invitrogen) at 150V for 75 minutes and transferred onto a PVDF membrane (manufactured by Millipore) using a semi-dry blotter. The PVDF membrane was blocked with a 5% ECL Blocking agent (manufactured by GE Healthcare) and the membrane was then incubated in a solution of anti-dystrophin antibody (manufactured by NCL-Dys1, Novocastra). After further incubation in a solution of peroxidase-conjugated goat-antimouse IgG (Model No. 170-6516, Bio-Rad), the membrane was stained with ECL Plus Western blotting system (manufactured by GE Healthcare).

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Immunostaining

The oligomer PMO No. 3 or 8 of the present invention was added to the cells. The cells after 72 hours were fixed in 3% paraformaldehyde for 10 minutes, followed by incubation in 10% Triton-X for 10 minutes. After blocking in 10% goat serum-containing PBS, the membrane was incubated in a solution of anti-dystrophin antibody (NCL-Dys1, Novocastra). The membrane was further incubated in a solution of anti-mouse IgG antibody (manufactured by Invitrogen). The membrane was mounted with Pro Long Gold Antifade reagent (manufactured by Invitrogen) and observed with a fluorescence microscope.

Experimental Results

The results are shown in FIGS. **6** and **7**. In this experiment it was confirmed by western blotting (FIG. **6**) and immunostaining (FIG. **7**) that the oligomers PMO Nos. 3 and 8 of the present invention induced expression of the dystrophin protein.

Test Example 5

In Vitro Assay Using Human Fibroblasts

The experiment was performed as in TEST EXAMPLE 3.

Experimental Results

The results are shown in FIG. **8**. This experiment revealed that in the cells from DMD patients with deletion of exons 45-52, the oligomers PMO Nos. 3 to 8 of the present invention caused exon 53 skipping with a higher efficiency than the oligomers PMO Nos. 13 and 14 of the present invention (FIG. **8**).

Test Example 6

In Vitro Assay

Experiments were performed using the antisense oligomers of 2'-O-methoxy-phosphorothioates (2-OMe-S-RNA) shown by SEQ ID NO: 49 to SEQ ID NO: 123. Various antisense oligomers used for the assay were purchased from Japan Bio Services. The sequences of various antisense oligomers are given below.

TABLE 7

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_39-69	CAUUCAACUGUUGCCUCCGGUUCUGAAGGUG	49
H53_1-25	UCCCACUGAUUCUGAAUUCUUUCAA	50
H53_6-30	CUUCAUCCCACUGAUUCUGAAUUCU	51
H53_11-35	UUGUACUUCAUCCCACUGAUUCUGA	52
H53_16-40	UGUUCUUGUACUUCAUCCCACUGAU	53
H53_21-45	GAAGGUGUUCUUGUACUUCAUCCCA	54
H53_26-50	GUUCUGAAGGUGUUCUUGUACUUCA	55
H53_31-55	CUCCGGUUCUGAAGGUGUUCUUGUA	56
H53_36-60	GUUGCCUCCGGUUCUGAAGGUGUUC	57
H53_41-65	CAACUGUUGCCUCCGGUUCUGAAGG	58
H53_46-70	UCAUUCAACUGUUGCCUCCGGUUCU	59
H53_51-75	ACAUUUCAUUCAACUGUUGCCUCCG	60
H53_56-80	CUUUAACAUUUCAUUCAACUGUUGC	61

TABLE 7-continued

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_61-85	GAAUCCUUUAACAUUUCAUUCAACU	62
H53_66-90	GUGUUGAAUCCUUUAACAUUUCAUU	63
H53_71-95	CCAUUGUGUUGAAUCCUUUAACAUU	64
H53_76-100	UCCAGCCAUUGUGUUGAAUCCUUUA	65
H53_81-105	UAGCUUCCAGCCAUUGUGUUGAAUC	66
H53_86-110	UUCCUUAGCUUCCAGCCAUUGUGUU	67
H53_91-115	GCUUCUUCCUUAGCUUCCAGCCAUU	68
H53_96-120	GCUCAGCUUCUUCCUUAGCUUCCAG	69
H53_101-125	GACCUGCUCAGCUUCUUCCUUAGCU	70
H53_106-130	CCUAAGACCUGCUCAGCUUCUUCCU	71
H53_111-135	CCUGUCCUAAGACCUGCUCAGCUUC	72
H53_116-140	UCUGGCCUGUCCUAAGACCUGCUCA	73
H53_121-145	UUGGCUCUGGCCUGUCCUAAGACCU	74
H53_126-150	CAAGCUUGGCUCUGGCCUGUCCUAA	75
H53_131-155	UGACUCAAGCUUGGCUCUGGCCUGU	76
H53_136-160	UUCCAUGACUCAAGCUUGGCUCUGG	77
H53_141-165	CCUCCUUCCAUGACUCAAGCUUGGC	78
H53_146-170	GGGACCCUCCUUCCAUGACUCAAGC	79
H53_151-175	GUAUAGGGACCCUCCUUCCAUGACU	80
H53_156-180	CUACUGUAUAGGGACCCUCCUUCCA	81
H53_161-185	UGCAUCUACUGUAUAGGGACCCUCC	82
H53_166-190	UGGAUUGCAUCUACUGUAUAGGGAC	83
H53_171-195	UCUUUUGGAUUGCAUCUACUGUAUA	84
H53_176-200	GAUUUUCUUUUGGAUUGCAUCUACU	85
H53_181-205	UCUGUGAUUUUCUUUUGGAUUGCAU	86
H53_186-210	UGGUUUCUGUGAUUUUCUUUUGGAU	87
H53_84-108	CCUUAGCUUCCAGCCAUUGUGUUGA	88
H53_88-112	UCUUCCUUAGCUUCCAGCCAUUGUG	89
H53_119-143	GGCUCUGGCCUGUCCUAAGACCUGC	90
H53_124-148	AGCUUGGCUCUGGCCUGUCCUAAGA	91
H53_128-152	CUCAAGCUUGGCUCUGGCCUGUCCU	92
H53_144-168	GACCCUCCUUCCAUGACUCAAGCUU	93
H53_149-173	AUAGGGACCCUCCUUCCAUGACUCA	94
H53_153-177	CUGUAUAGGGACCCUCCUUCCAUGA	95
H53_179-203	UGUGAUUUUCUUUUGGAUUGCAUCU	96
H53_184-208	GUUUCUGUGAUUUUCUUUUGGAUUG	97
H53_188-212	CUUGGUUUCUGUGAUUUUCUUUUGG	98
H53_29-53	CCGGUUCUGAAGGUGUUCUUGUACU	99

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TABLE 7-continued

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_30-54	UCCGGUUCUGAAGGUGUUCUUGUAC	100
H53_32-56	CCUCCGGUUCUGAAGGUGUUCUUGU	101
H53_33-57	GCCUCCGGUUCUGAAGGUGUUCUUG	102
H53_34-58	UGCCUCCGGUUCUGAAGGUGUUCUU	103
H53_35-59	UUGCCUCCGGUUCUGAAGGUGUUCU	104
H53_37-61	UGUUGCCUCCGGUUCUGAAGGUGUU	105
H53_38-62	CUGUUGCCUCCGGUUCUGAAGGUGU	106
H53_39-63	ACUGUUGCCUCCGGUUCUGAAGGUG	107
H53_40-64	AACUGUUGCCUCCGGUUCUGAAGGU	108
H53_32-61	UGUUGCCUCCGGUUCUGAAGGUGUUCUUGU	109
H53_32-51	GGUUCUGAAGGUGUUCUUGU	110
H53_35-54	UCCGGUUCUGAAGGUGUUCU	111
H53_37-56	CCUCCGGUUCUGAAGGUGUU	112
H53_40-59	UUGCCUCCGGUUCUGAAGGU	113
H53_42-61	UGUUGCCUCCGGUUCUGAAG	114
H53_32-49	UUCUGAAGGUGUUCUUGU	115
H53_35-52	CGGUUCUGAAGGUGUUCU	116
H53_38-55	CUCCGGUUCUGAAGGUGU	117
H53_41-58	UGCCUCCGGUUCUGAAGG	118
H53_44-61	UGUUGCCUCCGGUUCUGA	119
H53_35-49	UUCUGAAGGUGUUCU	120
H53_40-54	UCCGGUUCUGAAGGU	121
H53_45-59	UUGCCUCCGGUUCUG	122
H53_45-62	CUGUUGCCUCCGGUUCUG	123

RD cells (human rhabdomyosarcoma cell line) were plated at 3×10^5 in a 6-well plate and cultured in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37° C. and 5% CO_2 overnight. Complexes of various antisense oligomers (Japan Bio Services) (1 μM) for exon 53 skipping and Lipofectamine 2000 (manufactured by Invitrogen Corp.) were prepared and 200 μl was added to RD cells where 1.8 mL of the medium was exchanged, to reach the final concentration of 100 nM.

After completion of the addition, the cells were cultured overnight. The cells were washed twice with PBS (manufactured by Nissui, hereafter the same) and then 500 μl of ISOGEN (manufactured by Nippon Gene) were added to the cells. After the cells were allowed to stand at room temperature for a few minutes for cell lysis, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription
94° C., 2 mins: thermal denaturation
[94° C., 10 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification
68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3' (SEQ ID NO: 42)

Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3' (SEQ ID NO: 43)

Subsequently, a nested PCR was performed with the amplified product of RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94° C., 2 mins: thermal denaturation
[94° C., 15 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification
68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

Forward primer: 5'-AGGATTTGGAACAGAGGCGTC-3' (SEQ ID NO: 40)

Reverse primer: 5'-GTCTGCCACTGGCGGAGGTC-3' (SEQ ID NO: 41)

The reaction product, 1 μl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A+B)\times 100$$

Experimental Results

The results are shown in FIGS. **9** to **17**. These experiments revealed that, when the antisense oligomers were designed at exons 31-61 from the 5' end of exon 53 in the human dystrophin gene, exon 53 skipping could be caused with a high efficiency.

Test Example 7

Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 0.3 to 30 μM of the antisense oligomers were transfected with 3.5×10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

After the transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37° C. and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was then added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

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One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit (manufactured by Qiagen, Inc.). A reaction solution was prepared in accordance with the protocol attached to the kit. The thermal cycler used was a PTC-100 (manufactured by MJ Research). The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 30 seconds; 60° C., 30 seconds; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 10 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3' (SEQ ID NO: 42)

Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3' (SEQ ID NO: 43)

The reaction product, 1 μl, of the PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A+B) \times 100$$

Experimental Results

The results are shown in FIGS. **18** and **19**. These experiments revealed that the oligomer PMO No. 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomers PMO Nos. 15 and 16 (FIG. **18**). It was also revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the oligomers PMO Nos. 13 and 14 of the present invention (FIG. **19**). These results showed that the sequences with —OH group at the 5' end provide a higher skipping efficiency even in the same sequences.

INDUSTRIAL APPLICABILITY

Experimental results in TEST EXAMPLES demonstrate that the oligomers of the present invention (PMO Nos. 1 to 10) all caused exon 53 skipping with a markedly high efficiency under all cell environments, as compared to the oligomers (PMO Nos. 11, 12, 15 and 16) in accordance with the prior art. The 5017 cells used in TEST EXAMPLE 2 are the cells isolated from DMD patients, and the fibroblasts used in TEST EXAMPLES 3 and 5 are exon 53 skipping target cells from DMD patients. Particularly in TEST EXAMPLES 3 and 5, the oligomers of the present invention show the exon 53 skipping efficiency of 90% or higher in the cells from DMD patients that are the target for exon 53 skipping. Consequently, the oligomers of the present invention can induce exon 53 skipping with a high efficiency, when DMD patients are administered.

Therefore, the oligomers of the present invention are extremely useful for the treatment of DMD.

Sequence Listing Free Text

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SEQ ID NO: 3: synthetic nucleic acid
SEQ ID NO: 4: synthetic nucleic acid
SEQ ID NO: 5: synthetic nucleic acid
SEQ ID NO: 6: synthetic nucleic acid
SEQ ID NO: 7: synthetic nucleic acid
SEQ ID NO: 8: synthetic nucleic acid
SEQ ID NO: 9: synthetic nucleic acid
SEQ ID NO: 10: synthetic nucleic acid
SEQ ID NO: 11: synthetic nucleic acid
SEQ ID NO: 12: synthetic nucleic acid
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SEQ ID NO: 14: synthetic nucleic acid
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gagcactcgc acttccccgc ggcggtgcac ccggccccgg gcgcacgtga ggacgagcat 240

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tga 963

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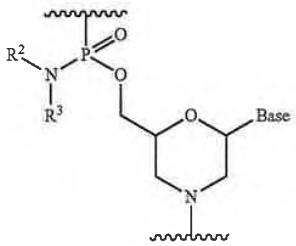
The invention claimed is:

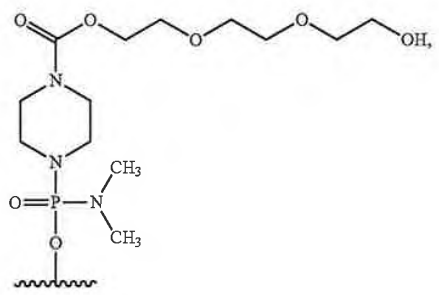
1. A solid-phase method of making an oligomer comprising a phosphorodiamidate morpholino oligomer (PMO) and a group at the 5' end of said PMO, wherein said PMO is 100% complementary to the 36th to the 60th nucleotides from the 5' end of the 53rd exon in a human dystrophin pre-mRNA, wherein the 53rd exon in said human dystrophin pre-mRNA consists of a nucleotide sequence corresponding to SEQ ID NO: 1, wherein said PMO hybridizes to said human dystrophin pre-mRNA with Watson-Crick base pairing, wherein the phosphorodiamidate morpholino monomers of said PMO have the formula:



wherein each of R^2 and R^3 represents a methyl;

wherein Base is a nucleobase selected from the group consisting of: uracil, cytosine, thymine, adenine, and guanine; and

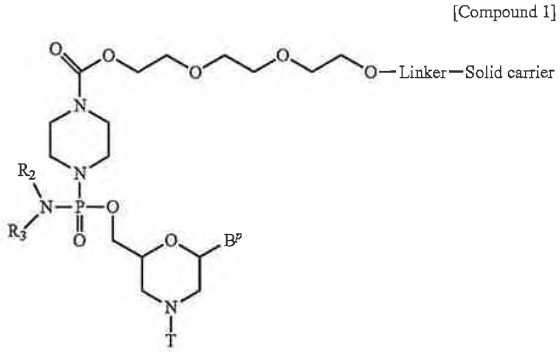
wherein the group at the 5' end of said PMO has the formula:



said method comprising:

a) providing Compound 1:

[Compound 1]



wherein T represents trityl, monomethoxytrityl, or dimethoxytrityl; wherein each of R^2 and R^3 represents a methyl; and wherein B^P is a protected Base,

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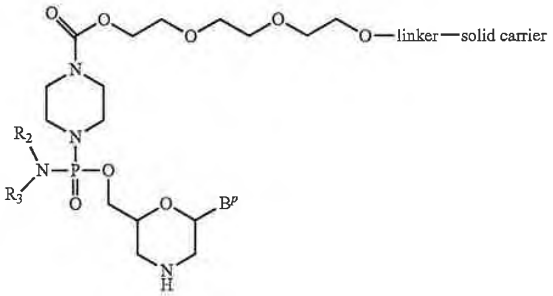
b) reacting said Compound 1 with an acid to form Compound 2;

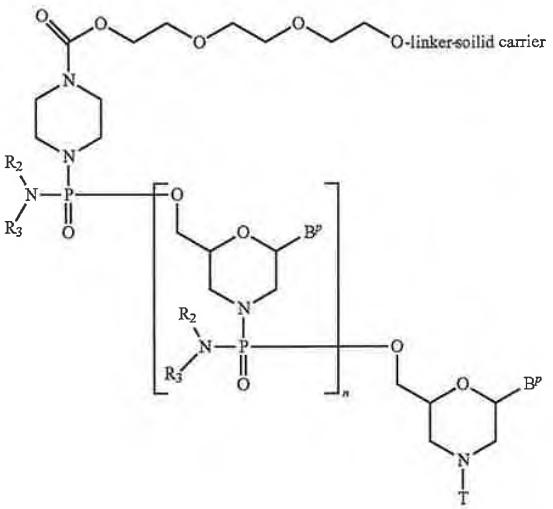
[Compound 2]



c) reacting said Compound 2 with a morpholino monomer in the presence of a base and a solvent;

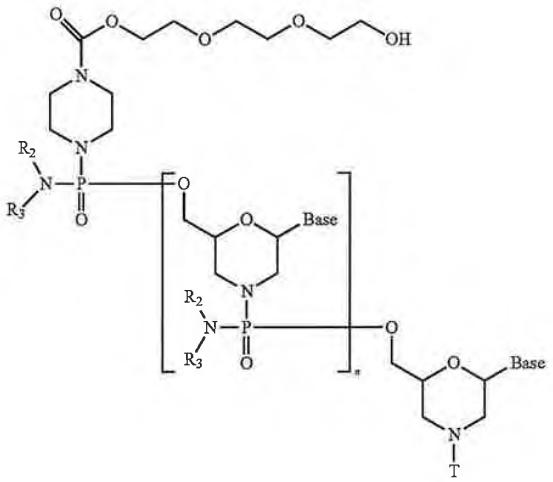
d) repeating steps b) and c) until Compound 3 is complete;

[Compound 3]



e) reacting said Compound 3 with a deprotecting agent to form Compound 4; and

[Compound 4]



f) reacting Compound 4 with an acid to form said oligomer.

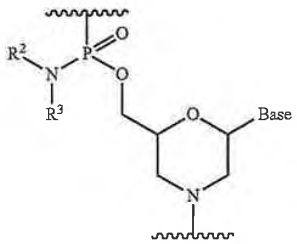
2. The method according to claim 1, wherein said acid used in step b) is trifluoroacetic acid.

3. The method according to claim 1, wherein said base used in step c) is N-ethylmorpholine and said solvent is N,N-dimethylimidazolidone.

4. The method according to claim 1, wherein said deprotecting agent is concentrated ammonia water used as a dilution with a solvent or a mixture of solvents.

5. The method according to claim 1, wherein said acid used in step f) is selected from phosphoric acid and hydrochloric acid.

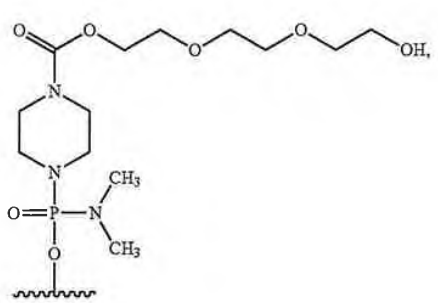
6. A solid-phase method of making a phosphorodiamidate morpholino oligomer (PMO) that is 100% complementary to the 36th to the 60th nucleotides from the 5' end of the 53rd exon in a human dystrophin pre-mRNA, wherein the 53rd exon in said human dystrophin pre-mRNA consists of a nucleotide sequence corresponding to SEQ ID NO: 1, wherein said PMO hybridizes to said human dystrophin pre-mRNA with Watson-Crick base pairing, wherein the phosphorodiamidate morpholino monomers of said PMO have the formula:



wherein each of R^2 and R^3 represents a methyl;

wherein Base is a nucleobase selected from the group consisting of: uracil, cytosine, thymine, adenine, and guanine; and

wherein the 5' end of said PMO has the formula:



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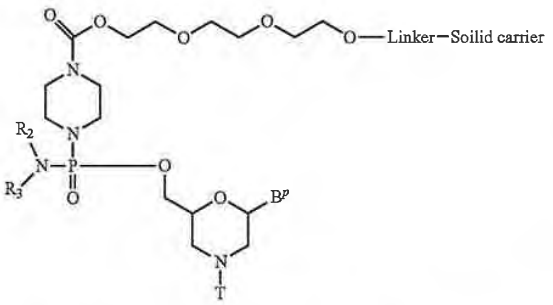
said method comprising:

a) providing Compound 1:

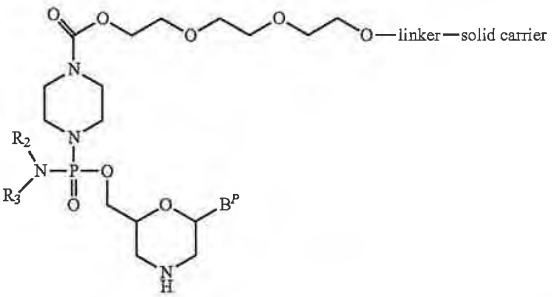
[Compound 1]



wherein T represents trityl, monomethoxytrityl, or dimethoxytrityl; wherein each of R^2 and R^3 represents a methyl; and wherein B^P is a protected Base;

b) reacting said Compound 1 with an acid to form Compound 2;

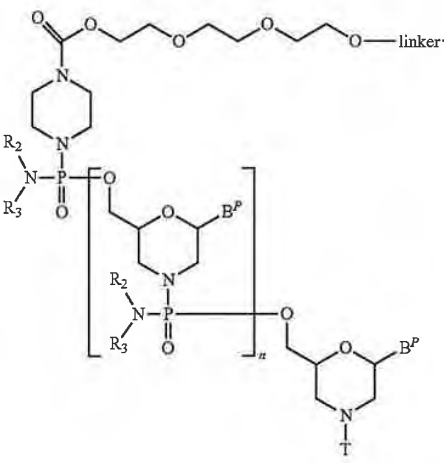
[Compound 2]



c) reacting said Compound 2 with a morpholino monomer in the presence of a base and a solvent;

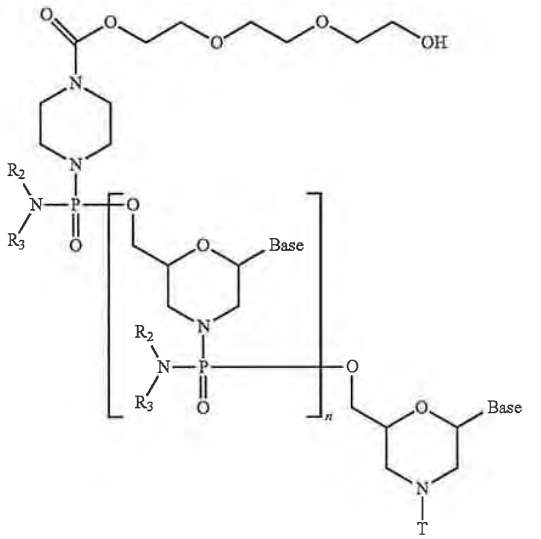
d) repeating steps b) and c) until Compound 3 is complete;

[Compound 3]

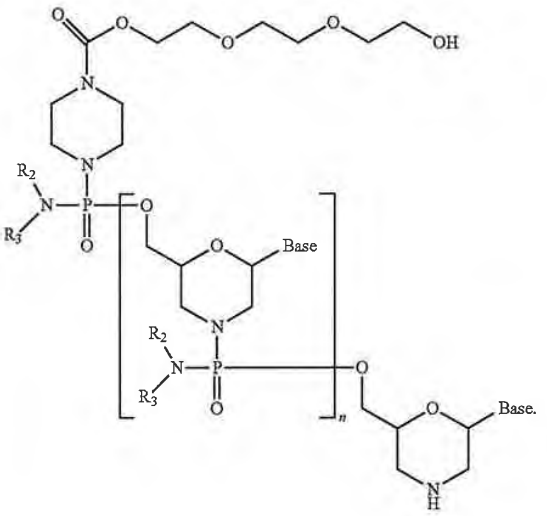


e) reacting said Compound 3 with a deprotecting agent to form Compound 4; and

[Compound 4]



f) reacting said Compound 4 with an acid to form said PMO:



7. The method according to claim **6**, wherein said acid used in step b) is trifluoroacetic acid.

8. The method according to claim **6**, wherein said base used in step c) is N-ethylmorpholine and said solvent is N,N-dimethylimidazolidone.

9. The method according to claim **6**, wherein said deprotecting agent is concentrated ammonia water used as a dilution with a solvent or a mixture of solvents.

10. The method according to claim **6**, wherein said acid used in step f) is selected from phosphoric acid and hydrochloric acid.

* * * * *

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U.S. DEPARTMENT OF COMMERCE
PATENT & TRADEMARK OFFICE

EXHIBIT 3

8145738

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

August 12, 2021

THIS IS TO CERTIFY THAT ANNEXED IS A TRUE COPY FROM THE RECORDS OF THIS OFFICE OF THE FILE WRAPPER AND CONTENTS OF:

APPLICATION NUMBER: *14/615,504*
FILING DATE: *February 06, 2015*
PATENT NUMBER: *9708361*
ISSUE DATE: *July 18, 2017*

Certified by

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

PTO/AIA/14 (12-13)
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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	209658-0001-01-US-518587
	Application Number	
Title of Invention	ANTISENSE NUCLEIC ACIDS	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Naoki		WATANABE	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Tsukuba-shi, Ibaraki	Country of Residence [i]	JP

Mailing Address of Inventor:

Address 1	Room 402, Ruvio II, 21-3, Sakura 1-chome		
Address 2			
City	Tsukuba-shi, Ibaraki	State/Province	
Postal Code	305-0003	Country [i]	JP

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Youhei		SATOU	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Tsukuba-shi, Ibaraki	Country of Residence [i]	JP

Mailing Address of Inventor:

Address 1	Room 402, Ruvio II, 21-3, Sakura 1-chome		
Address 2			
City	Tsukuba-shi, Ibaraki	State/Province	
Postal Code	305-0003	Country [i]	JP

Inventor 3 Remove

Legal Name

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	209658-0001-01-US-518587
	Application Number	
Title of Invention	ANTISENSE NUCLEIC ACIDS	

Prefix	Given Name	Middle Name	Family Name	Suffix
	Shin'ichi		TAKEDA	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Kodaira-shi, Tokyo	Country of Residence i	JP

Mailing Address of Inventor:

Address 1	c/o National Center of Neurology and Psychiatry		
Address 2	1-1, Ogawahigashicho 4-chome		
City	Kodaira-shi, Tokyo	**State/Province**	
Postal Code	187-8551	**Country** i	JP

Inventor 4 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Tetsuya		NAGATA	

Residence Information (Select One) ○ US Residency ◉ Non US Residency ○ Active US Military Service

City	Kodaira-shi, Tokyo	Country of Residence i	JP

Mailing Address of Inventor:

Address 1	c/o National Center of Neurology and Psychiatry		
Address 2	1-1, Ogawahigashicho 4-chome		
City	Kodaira-shi, Tokyo	**State/Province**	
Postal Code	187-8551	**Country** i	JP

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	055694		
Email Address	DBRIPDocket@dbr.com	Add Email	Remove Email

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	209658-0001-01-US-518587
	Application Number	
Title of Invention	ANTISENSE NUCLEIC ACIDS	

Application Information:

Title of the Invention	ANTISENSE NUCLEIC ACIDS		
Attorney Docket Number	209658-0001-01-US-518587	**Small Entity Status Claimed**	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	19	**Suggested Figure for Publication (if any)**	

Filing By Reference :

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	055694		

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	209658-0001-01-US-518587
	Application Number	
Title of Invention	ANTISENSE NUCLEIC ACIDS	

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this information in the application data sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the application number blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
	Continuation of	13891520	2013-04-10
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)
13891520	a 371 of international	PCT/JP2011/070318	2011-08-31

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button. Add

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55(d). When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX) the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(h)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Remove

Application Number	Country	Filing Date (YYYY-MM-DD)	Access Code (if applicable)
2010-196032	JP	2010-09-01	

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button. Add

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	209658-0001-01-US-518587
	Application Number	
Title of Invention	ANTISENSE NUCLEIC ACIDS	

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

Authorization to Permit Access:

☒ Authorization to Permit Access to the Instant Application by the Participating Offices

If checked, the undersigned hereby grants the USPTO authority to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the World Intellectual Property Office (WIPO), and any other intellectual property offices in which a foreign application claiming priority to the instant patent application is filed access to the instant patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JPO, KIPO, WIPO, or other intellectual property office in which a foreign application claiming priority to the instant patent application is filed to have access to the instant patent application.

In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the instant patent application with respect to: 1) the instant patent application-as-filed; 2) any foreign application to which the instant patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the instant patent application; and 3) any U.S. application-as-filed from which benefit is sought in the instant patent application.

In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date o f filing this Authorization.

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	209658-0001-01-US-518587
	Application Number	
Title of Invention	ANTISENSE NUCLEIC ACIDS	

Applicant 1 Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

◉ Assignee	○ Legal Representative under 35 U.S.C. 117	○ Joint Inventor
○ Person to whom the inventor is obligated to assign.	○ Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor :

If the Applicant is an Organization check here. ☒

Organization Name	NIPPON SHINYAKU CO., LTD.		
Mailing Address Information For Applicant:			
Address 1	14, Kisshoin Nishinosho Monguchicho, Minami-ku		
Address 2			
City	Kyoto-shi, Kyoto	**State/Province**	
Country [i]	JP	Postal Code	601-8550
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button. Add

Applicant 2 Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

◉ Assignee	○ Legal Representative under 35 U.S.C. 117	○ Joint Inventor
○ Person to whom the inventor is obligated to assign.	○ Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor :

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	209658-0001-01-US-518587
	Application Number	
Title of Invention	ANTISENSE NUCLEIC ACIDS	

If the Applicant is an Organization check here. ☒			
Organization Name	NATIONAL CENTER OF NEUROLOGY AND PSYCHIATRY		
Mailing Address Information For Applicant:			
Address 1	1-1, Ogawahigashicho 4-chome		
Address 2			
City	Kodaira-shi, Tokyo	**State/Province**	
Country i	JP	Postal Code	187-8551
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button. Add

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not subsitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication . An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

Remove

If the Assignee or Non-Applicant Assignee is an Organization check here. ☒			
Organization Name	NIPPON SHINYAKU CO., LTD.		
Mailing Address Information For Assignee including Non-Applicant Assignee:			
Address 1	14, Kisshoin Nishinosho Monguchicho, Minami-ku		
Address 2			
City	Kyoto-shi, Kyoto	**State/Province**	
Country i	JP	Postal Code	601-8550
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add

PTO/AIA/14 (12-13)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	209658-0001-01-US-518587
	Application Number	
Title of Invention	ANTISENSE NUCLEIC ACIDS	

Assignee 2

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication . An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

Remove

If the Assignee or Non-Applicant Assignee is an Organization check here. ☒

Organization Name	NATIONAL CENTER OF NEUROLOGY AND PSYCHIATRY

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1	1-1, Ogawahigashicho 4-chome		
Address 2			
City	Kodaira-shi, Tokyo	**State/Province**	
Country i	JP	Postal Code	187-8551
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add

Signature:

Remove

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Signature	/Zhengyu Feng/			Date (YYYY-MM-DD)	2015-02-06
First Name	Zhengyu	Last Name	Feng	Registration Number	66816

Additional Signature may be generated within this form by selecting the Add button. Add

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent C o o p eration Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Docket No.: 209658-0001-01-US-518587
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Naoki WATANABE et al.

Application No.: 14/615,504 | Confirmation No.: 2704

Filed: February 6, 2015 | Art Unit: N/A

For: ANTISENSE NUCLEIC ACIDS | Examiner: Not Yet Assigned

SECOND PRELIMINARY AMENDMENT

MS AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Madam:

Prior to examination on the merits, please amend the above-identified U.S. patent application as follows:

Amendments to the Claims begin on page 2.

Remarks begin on page 5.

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions, and listings, of the claims in the application:

LISTING OF CLAIMS

Claim 1. (Currently Amended): An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences consisting of: the 31st to the 55th, the 32nd to the 53rd, the 32nd to the 56th, the 32nd to the 61st, the 33rd to the 54th, the 33rd to the 57th, the 34th to the 58th, the 35th to the 59th, the 36th to the 53rd, the 36th to the 55th, the 36th to the 57th, the 36th to the 60th, or the 37th to the 61st ~~or the 36th to the 56th~~ nucleotides from the 5' end of the 53rd exon in the human dystrophin gene.

Claims 2-14. (Canceled).

Claim 15. (New): The antisense oligomer according to claim 1, which is an oligonucleotide.

Claim 16. (New): The antisense oligomer according to claim 15, wherein the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.

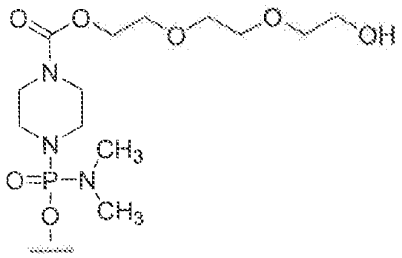
Claim 17. (New): The antisense oligomer according to claim 16, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of: OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br, and I, wherein R is an alkyl or an aryl and R' is an alkylene.

Claim 18. (New): The antisense oligomer according to claim 16, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of: a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond, and a boranophosphate bond.

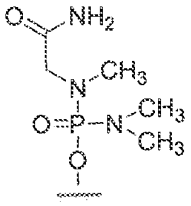
Claim 19. (New): The antisense oligomer according to claim 1, which is a morpholino oligomer.

Claim 20. (New): The antisense oligomer according to claim 19, which is a phosphorodiamidate morpholino oligomer.

Claim 21. (New): The antisense oligomer according to claim 19, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:



(1)



(2)

(3)

Claim 22. (New): The antisense oligomer according to claim 1, consisting of a nucleotide sequence complementary to the sequences consisting of the 32nd to the 56th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene.

Claim 23. (New): The antisense oligomer according to claim 1, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 4, 8, 11, 15, 18, 25, 32, 34, 36, 57, 103, 104, 105, and 109.

Claim 24. (New): The antisense oligomer according to claim 1, consisting of the nucleotide sequence shown by SEQ ID NO: 11.

Claim 25. (New): A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to claim 1, or a pharmaceutically acceptable salt or hydrate thereof.

REMARKS

Applicants herewith amend claim 1 and add new claims 15-25. Support for the amendments can be found at least from (1) the original claims of the PCT application, and (2) TEST EXAMPLE 7 and Table 7 of the Specification. No prohibited new matter is believed to be added. Applicants reserve the right to file a divisional or continuation application on any subject matter canceled by amendment. The cancelation of subject is without prejudice to, or disclaimer of, the subject matter.

CONCLUSION

If there are any other fees due in connection with the filing of this preliminary amendment, please charge the fees to our Deposit Account No. 50-0573. If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

Dated: February 9, 2015

Respectfully submitted,

By_____

Zhengyu Feng, Ph.D.
Registration No.: 66,816
DRINKER BIDDLE & REATH LLP
1500 K Street, N.W.
Suite 1100
Washington, DC 20005-1209
202.230.5119 (Phone)
202.842.8465 (Fax)
Attorneys/Agents For Applicant

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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EXAMINER	
MCGARRY, SEAN	

ART UNIT	PAPER NUMBER
1674	

NOTIFICATION DATE	DELIVERY MODE
03/25/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

DBRIPDocket@dbr.com
penelope.mongelluzzo@dbr.com

Office Action Summary	Application No. 14/615,504	Applicant(s) WATANABE ET AL.	
	Examiner SEAN MCGARRY	Art Unit 1674	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1 and 15-25* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1 and 15-25* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Claim Rejections - 35 USC § 101

35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Claims 1, 15, and 22-25 are rejected under 35 U.S.C. 101 because the claimed invention is not directed to patent eligible subject matter. Based upon an analysis with respect to the claim as a whole, the claims do not recite something significantly different than a judicial exception. The claimed invention reads on a fragment of a naturally occurring nucleic acid.

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

Claims 1-8 and 12-14 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Popplewell et al [US20100168212], Sazani et al [US20100130591] in view of Baker et al [US20130109091] and Bennett et al [US20120190728].

The claimed invention is drawn to antisense compounds targeted to recited regions all contained within nucleotides 31-61 of exon 53 of the dystrophin gene including antisense oligomers SEQ ID NOS:4, 8, 11, 15, 18, 25, 32, 34, 36, 57, 103,-105, and 109 that cause skipping of the 53rd exon in the human dystrophin gene. The invention includes modifications to the compounds where these modifications are well known and routinely utilized in the antisense art at the time of invention.

Popplewell et al have taught antisense based alteration of splicing in the human dystrophin gene including use as pharmaceuticals. It has been taught to target exon 53 to induce skipping of the 53rd exon. The specific sequences and modifications recited in the instant claims have been clearly suggested by Popplewell et al. See for example SEQ ID NOS:10-12 and 24, and paragraph 15:

The molecule that causes skipping in exon 53 comprises at least a 25 base length from a base sequence selected from:

TABLE-US-00005 (SEQ ID NO: 10) j) CXG XXG CCX CCG GXX CXG AAG GXG XXC XXG; (SEQ ID NO: 11) k) CAA CXG XXG CCX CCG GXX CXG AAG GXG XXC; or (SEQ ID NO: 12) l) XXG CCX CCG GXX CXG AAG GXG XXC XXG XAC,

wherein the molecule's sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in exon 53 of the dystrophin gene.

Paragraph 28:

The base "X" in the above base sequences is defined as being thymine (T) or uracil (U). The presence of either base in the sequence will still allow the molecule to bind to the pre-mRNA of the dystrophin gene as it is a complementary sequence. Therefore, the presence of either base in the molecule will cause exon skipping. The base sequence of the molecule may

contain all thymines, all uracils or a combination of the two. One factor that can determine whether X is T or U is the chemistry used to produce the molecule. For example, if the molecule is a phosphorodiamidate morpholino oligonucleotide (PMO), X will be T as this base is used when producing PMOs. Alternatively, if the molecule is a phosphorothioate-linked 2'-O-methyl oligonucleotide (2'OMePS), X will be U as this base is used when producing 2'OMePSs. Preferably, the base "X" is only thymine (T).

Paragraph 30:

The molecule can be any type of molecule as long as it has the selected base sequence and can bind to a target site of the dystrophin pre-mRNA to cause exon skipping. For example, the molecule can be an oligodeoxyribonucleotide, an oligoribonucleotide, a phosphorodiamidate morpholino oligonucleotide (PMO) or a phosphorothioate-linked 2'-O-methyl oligonucleotide (2'OMePS). Preferably, the oligonucleotide is a PMO. The advantage of a PMO is that it has excellent safety profiles and appears to have longer lasting effects in vivo compared to 2'OMePS oligonucleotides. Preferably, the molecule is isolated so that it is free from other compounds or contaminants.

Paragraph 32:

The molecule is at least 25 bases in length. Preferably, the molecule is at least 28 bases in length. Preferably, the molecule is no more than 35 bases in length and, more preferably, no more than 32 bases in length. Preferably, the molecule is between 25 and 35 bases in length, more preferably, the molecule is between 28 and 32 bases in length, even more preferably, the molecule is between 29 and 31 bases in length, and most preferably, the molecule is 30 bases in length. It has been found that a molecule which is 30 bases in length causes efficient exon skipping. If the molecule is longer than 35 bases in length, the specificity of the binding to the specific exon region is reduced. If the molecule is less than 25 bases in length, the exon skipping efficiency is reduced.

Paragraph 96:

To ensure that the analysis of PMOs for the targeted skipping of exon 53 was not biased by any particular design strategy, seventeen 25mer PMOs were designed to cover the whole of exon 53, with stepwise arrays over suggested bioactive target sites, and then subsequently six 30mer PMOs were designed to target the sequence of exon 53 that showed an association with exon skipping for the 25mers tested. PMOs were designed and tested independently by two different groups (at RHUL and UWA), and then efficacy of the best thirteen sequences confirmed by two other independent groups (at UCL and LUMC). Such a collaborative approach has been used previously as a way of validating target sequences in DMD [4]. Human myoblasts allowed the controlled in vitro comparison of PMO sequences, and confirmation of skipping of

exon 53 at the RNA level by certain PMOs in both normal cells and, perhaps more importantly, in DMD patient cells with a relevant mutation. These results were further borne out by the expression of dystrophin protein in the DMD cells treated with specific PMOs. Use of the humanised DMD mouse provided an in vivo setting to confirm correct exon exclusion prior to any planned clinical trial. The combined use of these three different systems (normal cells, patient cells and hDMD mouse) as tests of PMO bioactivity provided a reliable and coherent determination of optimal sequence(s) for the targeted skipping of exon 53.

Paragraph 97:

When considering the data presented here as a whole, the superiority of the PMO targeting the sequence +30+59 (PMO-G, or h53A30/1), is strongly indicated. In normal myoblasts, nucleofection of PMO-G (300 nM) and liposomal-carrier mediated transfection of leashed PMO-G (500 nM) produced over 80% and over 50% skipping of exon 53, respectively, implying that it acts extremely efficiently within the cell. This was confirmed in patient cells. Indeed, this PMO generates the highest levels of exon skipping in patient cells over a range of concentrations (up to 200 nM) and, most important therapeutically, exerts its activity at concentrations as low as 25 nM. The exon skipping activity of this PMO is also persistent, with over 70% exon skipping for 7 days in culture, and over 60% exon skipping for up to three weeks. This would have important safety and cost implications as a genetic therapy for DMD patients with the appropriate deletions. PMO-G was also shown to skip exon 53 correctly in vivo. These RNA results were further confirmed by the detection of dystrophin protein at a high level in protein extracts from patient cells treated with PMO-G. Previous studies by the Leiden group [18] suggest that the optimal 2'OMePS AO is targeted to the sequence +46+63 of exon 53, producing exon skipping in up to 25% of transcripts in cultured cells and 7% in the hDMD mouse. This 2'OMePS AO shows some degree of overlap with the optimal PMOs reported here which strengthens our findings. The reason that our optimal PMO is more specific could be a (combined) consequence of the different AO chemistries, length of AO used, and the absolute target site of AO.

The prior art has therefore taught that the same region targeted by the instantly claimed oligomers is superior to other regions of exon 53. The prior art has taught that sequences with SEQ ID NOS:10-12 are included in their invention. The recited SEQ ID NOS: fall squarely within SEQ ID NOS:10-12 and 24 which has been taught by Popplewell to be a “superior” target region of exon 53. While the entire document is pertinent to applicant invention, please also see Example 2 and claims 1-12.

Sazani et al have also taught antisense oligomers for inducing exon 53 skipping in the human dystrophin gene. Sazani et al have also taught oligomers targeting the same target site and the instant invention and the superior region taught by Popplewell et all See for example SEQ ID NOS: 430, 431, and 628-633 which all overlap or is/are embrace the instantly recited SEQ ID NOS. Sazani et al have also taught that oligomer size choices and modification of antisense oligonucleotides. while the entire reference is relied upon and relevant to applicants invention, applicant is directed to, for example, paragraphs 18-25, 36, 40, 50, 56, 95, 97, 98, 104, 118, 123-177, 196, 197, and claims 36-39, for example.

While the prior art has not specifically disclosed the recited sequences SEQ ID Nos, the prior art has clearly taught that such sequences are embraced within a known target region and furthermore within known antisense compounds. The prior art, however, has taught that the region that the instant compounds are targeted to is a superior target region and furthermore the prior art references taken together have taught that one in the art can alter the sizes of the antisense compounds. It would have been well within the skill of the artisan to test various sizes of oligomers in an optimization of antisense compounds targeting a known superior target region. It is noted that the superior target region is not large; "When considering the data presented here as a whole, the superiority of the PMO targeting the sequence +30+59 (PMO-G, or h53A30/1), is strongly indicated." Applicants invention is oligomers that are within this exemplified

compound where it has been clearly taught that sequences within this oligomer were considered y by the prior art. The modifications utilized in the invention and recited in the claims were well known and routinely used in the art at the time of invention as shown by the above art and evidenced by Baker et al and Bennett et al. The benefits of the modifications were well known in the art where nuclease protection, and improved hybridization, and cell penetration were known benefits, for example. Both of these references are drawn to antisense compounds utilized in alteration of splicing. See Paragraphs 10, 11, 13, 27, and 60-71 of Baker et al and paragraphs 25, 57-75, 97-104, 140-155, 176, and 180-183 of Bennett et al, for example. Bennett and Baker et al have also taught various size ranges for splice altering antisense compounds.

The invention as a whole would therefore have been *prima facie* obvious to one in the art at the time of invention.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the “right to exclude” granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the claims at issue are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s)

because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); In re Vogel, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the reference application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/forms/. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more

information about eTerminal Disclaimers, refer to

http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

Claims 1 and 15-25 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-7 of U.S. Patent No. 9079934. Although the claims at issue are not identical, they are not patentably distinct from each other because the antisense oligomer of the patent overlaps significantly with and within antisense compounds targeted within or to the regions recited in the instant claim1, for example.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to SEAN MCGARRY whose telephone number is (571)272-0761. The examiner can normally be reached on M-Th (7:00-5:30).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Shaojia Anna Jiang can be reached on (571) 272-0627. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Sean R McGarry
Primary Examiner
Art Unit 1674

/SEAN MCGARRY/
Primary Examiner, Art Unit 1674

Docket No.: 209658-0001-01-US-518587
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Naoki WATANABE et al.

Application No.: 14/615,504 Confirmation No.: 2704

Filed: February 6, 2015 Art Unit: 1674

For: ANTISENSE NUCLEIC ACIDS Examiner: S. McGarry

AMENDMENT / RESPONSE UNDER 37 C.F.R. § 1.111
&
PETITION FOR EXTENSION OF TIME

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the Office Action mailed March 25, 2016, Applicants submit the present Amendment / Response. The Office is respectfully requested to consider and enter the following amendments and remarks. Applicants petition herewith for a ONE-month extension of time and submit the corresponding fee, extending the period of response until July 25, 2016.

Amendments to the Abstract begin on page 2.

Amendments to the Claims begin on page 4.

Remarks begin on page 6.

AMENDMENTS TO THE ABSTRACT

Please replace the Abstract with the Substitute Abstract provided on the next page.

SUBSTITUTE ABSTRACT

~~The present invention provides a pharmaceutical composition which causes skipping of the 53rd exon in the human dystrophin gene with a high efficiency.~~

The present invention provides an oligomer which efficiently enables to cause skipping of the 53rd exon in the human dystrophin gene. Also provided is a pharmaceutical composition which causes skipping of the 53rd exon in the human dystrophin gene with a high efficiency.

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions, and listings, of the claims in the application:

LISTING OF CLAIMS

Claim 1. (Currently Amended): An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of [[a]]the nucleotide sequence ~~complementary to any one of the sequences consisting of: the 31st to the 55th, the 32nd to the 53rd, the 32nd to the 56th, the 32nd to the 61st, the 33rd to the 54th, the 33rd to the 57th, the 34th to the 58th, the 35th to the 59th, the 36th to the 53rd, the 36th to the 55th, the 36th to the 57th, the 36th to the 60th, or the 37th to the 61st nucleotides from the 5' end of the 53rd exon in the human dystrophin gene~~of SEQ ID NO: 11 and SEQ ID NO: 57, wherein the antisense oligomer is an oligonucleotide in which the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified, or a morpholino oligomer .

Claims 2-16. (Canceled).

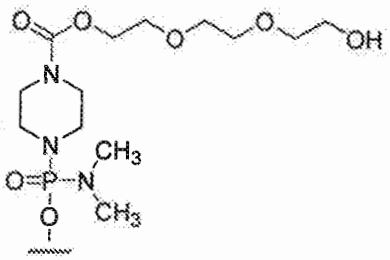
Claim 17. (Currently Amended): The antisense oligomer according to claim [[16]]1, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of: OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br, and I, wherein R is an alkyl or an aryl and R' is an alkylene.

Claim 18. (Currently Amended): The antisense oligomer according to claim [[16]]1, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of: a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond, and a boranophosphate bond.

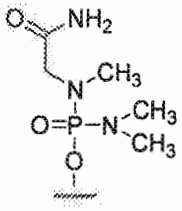
Claim 19. (Previously Presented): The antisense oligomer according to claim 1, which is a morpholino oligomer.

Claim 20. (Previously Presented): The antisense oligomer according to claim 19, which is a phosphorodiamidate morpholino oligomer.

Claim 21. (Previously Presented): The antisense oligomer according to claim 19, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:



(1)



(2)

(3)

Claim 22. (Canceled).

Claim 23. (Currently Amended): The antisense oligomer according to claim 1, consisting of the nucleotide sequence ~~shown by any one selected from the group consisting of SEQ ID NOS: 4, 8, 11, 15, 18, 25, 32, 34, 36, 57, 103, 104, 105, and 109~~of SEQ ID NO: 57.

Claim 24. (Currently Amended): The antisense oligomer according to claim 1, consisting of the nucleotide sequence ~~shown by~~of SEQ ID NO: 11.

Claim 25. (Previously Presented): A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to claim 1, or a pharmaceutically acceptable salt or hydrate thereof.

REMARKS

Applicants request reconsideration in light of the above amendments and following comments submitted under 37 C.F.R. § 1.111.

1. Amendments to the Abstract

Applicants amend the Abstract to a single paragraph. No prohibited new matter is believed to be introduced.

2. Status of the Claims

The status of the claims following entry of the amendments is as follows:

Claims pending: 1, 17-21, and 23-25

Claims rejected: 1 and 15-25

Claims canceled: 2-16 and 22

Claims amended: 1, 17-18, and 22-23

Applicants amend claim 1 to recite SEQ ID NO: 11 and SEQ ID NO: 57, which respectively correspond to nucleotide sequences complementary to 36th to 60th and 32nd to 56th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene. Applicants also amend claims 17-18 to update the dependency given the cancellation of claim 16. Applicants further amend claims 22 and 23 to recite SEQ ID NO: 57 and SEQ ID NO: 11, respectively. Thus, no prohibited new matter is believed to be added.

The claims have been amended without prejudice to, or disclaimer of, the canceled subject matter. Applicant reserves the right to file a continuation or divisional application on any subject matter canceled by way of amendments.

3. Information Disclosure Statements

Applicants appreciate the Office's acknowledgement of the Information Disclosure Statements (IDSs) submitted on February 6, 2015, and September 22, 2015.

4. Status of the Drawings

Applicants respectfully request status as to the acceptance of the drawings as filed February 6, 2015 and Replacement Sheets March 3, 2016 with the Office's next communication.

5. Priority Documents

Applicants respectfully request acknowledgment of the claim for foreign priority and receipt of the priority document(s) with the Office's next communication.

6. Claim Rejection Under 35 U.S.C. § 101

The Office rejects claims 1, 15, and 22-25 as allegedly not directed to patent eligible subject matter. Office Action, page 2. The Office alleges that (1) "the claims do no recite something significantly different than a judicial exception"; and (2) "[t]he claimed invention reads on a fragment of a naturally occurring nucleic acid." *Id.*

Upon entry of the present amendments, claims 15 and 22 stand canceled, mooting at least this aspect of the rejection. Without acquiescing as to the merits of the Office's rejection, amended independent claim 1 recites, *inter alia*, an antisense oligomer that is either (1) a modified oligonucleotide (in which the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide has been modified), or (2) a morpholino oligomer.[1] There is no evidence on the record, or adduced by the Office, that any one of the presently recited antisense oligomers would have existed as "a fragment of a naturally occurring nucleic acid." The Office's rejection is thus moot. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of claims 1 and 23-25.

7. Claim Rejection Under 35 U.S.C. § 103(a)

The Office rejects claims 1 and 15-25[2] as allegedly obvious over **Popplewell** et al., US 2010/0168212 ("Popplewell") and **Sazani** et al., US 2010/0130591 ("Sazani") in view of **Baker**

[1] Morpholino oligomers are synthetic molecules having standard nucleic acid bases bound to morpholine rings (instead of the deoxyribose rings in DNA). *See, e.g.*, Wikipedia page of Morpholino, *available at* http://en.wikipedia.org/wiki/Morpholino.

[2] The Office alleges "[c]laims 1-8 and 12-14" as be unpatentable over the cited references. Applicants believe that the Office must have meant "claims 1 and 15-25" as indicated in the Office Action Summary (PTOL-326).

et al., US 2013/0109091 ("Baker") and **Bennett** et al., US 2012/0190728 ("Bennett"). Office Action, pages 3-8.

Alleged Grounds for Rejection

Popplewell allegedly teaches targeting the 53rd exon of the human dystrophin gene to induce skipping of the 53rd exon. *Id.*, at 4. Popplewell's SEQ ID NOs: 10-12 and 24 allegedly suggest the presently recited sequences and modifications. *Id.*

Sazani allegedly teaches antisense oligomers for inducing exon 53 skipping in the human dystrophin gene. *Id.*, at 7. Sazani's SEQ ID NOs: 430-431 and 628-633 allegedly overlap or encompass the presently recited SEQ ID NOs: 11 or 35. *Id.*

The Office admits that none of the cited references discloses the presently recited SEQ ID NOs. *Id.*, at 8. Nevertheless, the Office alleges that "[i]t would have been well within the skill of the artisan to test various sizes of oligomers in an optimization of antisense compounds targeting a known superior target region." *Id.*

The Office by relying upon Baker and Bennett further alleges that "[t]he modifications utilized in the invention and recited in the claims were well known and routinely used in the art at the time." *Id.*, 8. The Office then concludes that "[t]he invention as whole would therefore have been prima facie obvious to one in the art at the time of invention." *Id.*

Applicants traverse the rejection to the extent it may be applied to the amended claims. Both the suggestion of the claimed invention and the expectation of success must be in the prior art, not in the disclosure of the claimed invention. *In re Dow Chem. Co.*, 837 F.2d 469 (Fed. Cir. 1988). Additionally, once the scope and content of the prior art are determined, the relevant inquiry is whether the prior art suggests all the limitations of the invention, and whether one of ordinary skill in the art would have had a reasonable expectation that the claimed invention would be successful as so combined. *In re Vaeck*, 947 F.2d 488, 493 (Fed. Cir. 1991).

Upon entry of the present amendments, independent claim 1 recites, *inter alia*, an oligomer consisting of the nucleotide sequence of SEQ ID NO: 11 or SEQ ID NO: 57. As the Office admits, none of the cited references teaches or suggests SEQ ID NO: 11 or SEQ ID NO: 57. The Office's rejection is unsupported, at least because the Office fails to articulate a rationale why a skilled artisan would have been guided or directed to modify the antisense oligomers of Sazani to arrive at the presently claimed antisense oligomers. Without such

guidance, the artisan would not have had a reasonable expectation of success in arriving at the claimed sequences. *See, e.g., In re O'Farrell*, 853 F.2d 894, 903 (Fed. Cir. 1988).[3] Teachings of Baker and Bennett are not directly applicable, because the targeted genes discussed therein differ from the human dystrophin gene.

Additionally, the presently recited oligomers (consisting of the nucleotide sequence of SEQ ID NO: 11 and 57) offer superior skipping effects over the oligomers taught in both Popplewell and Sazani. For example, Figures 2-4 of the Specification (corresponding to data in Test Examples 2-3) show that PMO No. 3 (having the nucleotide sequence of SEQ ID NO: 11; *see* Table 2) outperformed exemplary antisense oligomers taught in Popplewell (PMO Nos. 12 and 15). Additionally, Figures 18-19 of the Specification (corresponding to data in Test Example 7) show that PMO No. 3 (having the nucleotide sequence of SEQ ID NO: 11; *see* Table 2) outperformed exemplary antisense oligomer taught in Sazani (PMO No. 16).[4] Furthermore, Figures 16-17 (corresponding to data in Test Example 6) show that the oligomer having the nucleotide sequence of SEQ ID NO: 57 (H53_36-60) displays a higher level skipping activity that that having the nucleotide sequence of SEQ ID NO: 11 (H53_32-56). Thus, the recited oligomers consisting of the nucleotide sequence of SEQ ID NO: 57 also have superior skipping activity over exemplary oligomers taught in Popplewell and Sazani. Applicants submit that this superiority is unexpected, at least because none of the cited references teach or suggest such an effect.

Given at least the above arguments, claim 1 as amended and its dependent claims 17-21, and 23-25 would have been nonobvious over cited references. Claims 15-16 and 22 stand canceled, mooting at least this aspect of the rejection. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of the claims.

---

[3] "The admonition that 'obvious to try' is not the standard under § 103 has been directed mainly at two kinds of error. In some cases, what would have been 'obvious to try' would have been to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful."

[4] Figure 19 shows that PMO No. 3 has an equivalent level of skipping activity as PMO No. 8. Figure 18 shows that PMO No. 8 has a higher level of skipping activity than the exemplary antisense oligomer taught in Sazani (PMO No. 16). Thus, a skilled artisan given Figures 18-19 would have understood the following order of the skipping activities:

PMO No. 3 ≈ PMO No. 8 >> PMO No. 16.

8. Double Patenting Rejection

The Office rejects claims 1 and 15-25 on the ground of nonstatutory double patenting as allegedly obvious over claims 1-7 of **U.S. Patent No. 9,079,934** ("the '934 patent"). Office Action, pages 8-10. The Office alleges:

> Although the claims at issue are not identical, they are not patentably distinct from each other because the antisense oligomer of the patent overlaps significantly with and within antisense compounds targeted within or to the regions recited in the instant claim 1, for example.

Id., at 10.

Without acquiescing as to the merits of the Office's rejection, Applicants amend claim 1 to recite, *inter alia*, an oligomer consisting of the nucleotide sequence of SEQ ID NO: 11 or SEQ ID NO: 57. SEQ ID NO: 11 corresponds to H53_32-56, while SEQ ID NO: 57 corresponds to H53_36-60. The '934 patent recites oligomers consisting of the nucleotide sequence of SEQ ID NO: 35, which correspond to H53_36-56. In addition to the sequence differences, the '934 patent's oligomers are shorter (21-mer) that the presently claimed oligomers (25-mer). Applicants respectfully request the Office's reconsideration given the present claim amendments.

If necessary, Applicants may consider submitting a Terminal Disclaimer Form and payment of the appropriate fee, when the obviousness-type double patenting rejection becomes the only outstanding rejection remaining.

CONCLUSION

In view of the foregoing, Applicant submits that the pending claims are in condition for allowance, and respectfully request reconsideration and timely allowance of the pending claims. Should the Examiner feel that there are any issues outstanding after consideration of this response; the Examiner is invited to contact Applicant's undersigned representative to expedite prosecution. A favorable action is awaited.

EXCEPT for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. § 1.16 and 1.17 which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account No. 50-0573. This paragraph is intended to be a CONSTRUCTIVE PETITION FOR EXTENSION OF TIME in accordance with 37 C.F.R. § 1.136(a)(3).

Dated: July 22, 2016

Respectfully submitted,

Customer Number: 055694

By /Zhengyu Feng/
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/615,504	02/06/2015	Naoki WATANABE	209658-0001-01-US-518587	2704

55694 7590 10/27/2016

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EXAMINER	
MCGARRY, SEAN	

ART UNIT	PAPER NUMBER
1674	

NOTIFICATION DATE	DELIVERY MODE
10/27/2016	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

DBRIPDocket@dbr.com
penelope.mongelluzzo@dbr.com

Office Action Summary	Application No. 14/615,504	Applicant(s) WATANABE ET AL.	
	Examiner SEAN MCGARRY	Art Unit 1674	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on *7/22/2016*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5)☒ Claim(s) *1,17-21,24 and 25* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1,17-21,24 and 25* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on *2/6/2015 and 3/3/2015* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☒ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☒ Certified copies of the priority documents have been received in Application No. 13/819,520.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Claim Rejections - 35 USC § 101

35 U.S.C. 101 reads as follows:

> Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

Claims 1, 15, and 22-25 **WERE** rejected under 35 U.S.C. 101 because the claimed invention is not directed to patent eligible subject matter. Based upon an analysis with respect to the claim as a whole, the claims do not recite something significantly different than a judicial exception.

This rejection has been withdrawn in view of applicants amendments to the claims filed 7/22/2016.

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which

said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

Claims 1, 17-21 and 23-25 are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Popplewell et al [US20100168212], Sazani et al [US20100130591] in view of Baker et al [US20130109091] and Bennett et al [US20120190728].

The claimed invention is drawn to antisense compounds targeted to recited regions all contained within nucleotides 31-61 of exon 53 of the dystrophin gene including antisense oligomers SEQ ID NOS:4, 8, 11, 15, 18, 25, 32, 34, 36, 57, 103,-105, and 109 that cause skipping of the 53rd exon in the human dystrophin gene. The invention includes modifications to the compounds where these modifications are well known and routinely utilized in the antisense art at the time of invention.

Popplewell et al have taught antisense based alteration of splicing in the human dystrophin gene including use as pharmaceuticals. It has been taught to target exon 53 to induce skipping of the 53rd exon. The specific sequences and modifications recited in the instant claims have been clearly suggested by Popplewell et al. See for example SEQ ID NOS:10-12 and 24, and paragraph 15:

The molecule that causes skipping in exon 53 comprises at least a 25 base length from a base sequence selected from:

TABLE-US-00005 (SEQ ID NO: 10) j) CXG XXG CCX CCG GXX CXG AAG GXG XXC XXG; (SEQ ID NO: 11) k) CAA CXG XXG CCX CCG GXX CXG AAG GXG XXC; or (SEQ ID NO: 12) l) XXG CCX CCG GXX CXG AAG GXG XXC XXG XAC,

wherein the molecule's sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in exon 53 of the dystrophin gene.

Paragraph 28:

The base "X" in the above base sequences is defined as being thymine (T) or uracil (U). The presence of either base in the sequence will still allow the molecule to bind to the pre-mRNA of the dystrophin gene as it is a complementary sequence. Therefore, the presence of either base in the molecule will cause exon skipping. The base sequence of the molecule may contain all thymines, all uracils or a combination of the two. One factor that can determine whether X is T or U is the chemistry used to produce the molecule. For example, if the molecule is a phosphorodiamidate morpholino oligonucleotide (PMO), X will be T as this base is used when producing PMOs. Alternatively, if the molecule is a phosphorothioate-linked 2'-O-methyl oligonucleotide (2'OMePS), X will be U as this base is used when producing 2'OMePSs. Preferably, the base "X" is only thymine (T).

Paragraph 30:

The molecule can be any type of molecule as long as it has the selected base sequence and can bind to a target site of the dystrophin pre-mRNA to cause exon skipping. For example, the molecule can be an oligodeoxyribonucleotide, an oligoribonucleotide, a phosphorodiamidate morpholino oligonucleotide (PMO) or a phosphorothioate-linked 2'-O-methyl oligonucleotide

(2'OMePS). Preferably, the oligonucleotide is a PMO. The advantage of a PMO is that it has excellent safety profiles and appears to have longer lasting effects in vivo compared to 2'OMePS oligonucleotides. Preferably, the molecule is isolated so that it is free from other compounds or contaminants.

Paragraph 32:

The molecule is at least 25 bases in length. Preferably, the molecule is at least 28 bases in length. Preferably, the molecule is no more than 35 bases in length and, more preferably, no more than 32 bases in length. Preferably, the molecule is between 25 and 35 bases in length, more preferably, the molecule is between 28 and 32 bases in length, even more preferably, the molecule is between 29 and 31 bases in length, and most preferably, the molecule is 30 bases in length. It has been found that a molecule which is 30 bases in length causes efficient exon skipping. If the molecule is longer than 35 bases in length, the specificity of the binding to the specific exon region is reduced. If the molecule is less than 25 bases in length, the exon skipping efficiency is reduced.

Paragraph 96:

To ensure that the analysis of PMOs for the targeted skipping of exon 53 was not biased by any particular design strategy, seventeen 25mer PMOs were designed to cover the whole of exon 53, with stepwise arrays over suggested bioactive target sites, and then subsequently six 30mer PMOs were designed to target the sequence of exon 53 that showed an association with exon skipping for the 25mers tested. PMOs were designed and tested independently by two different groups (at RHUL and UWA), and then efficacy of the best thirteen sequences confirmed by two other independent groups (at UCL and LUMC). Such a collaborative approach has been used previously as a way of validating target sequences in DMD [4]. Human myoblasts allowed the controlled in vitro comparison of PMO sequences, and confirmation of skipping of exon 53 at the RNA level by certain PMOs in both normal cells and, perhaps more importantly, in DMD patient cells with a relevant mutation. These results were further borne out by the expression of dystrophin protein in the DMD cells treated with specific PMOs. Use of the humanised DMD mouse provided an in vivo setting to confirm correct exon exclusion prior to any planned clinical trial. The combined use of these three different systems (normal cells, patient cells and hDMD mouse) as tests of PMO bioactivity provided a reliable and coherent determination of optimal sequence(s) for the targeted skipping of exon 53.

Paragraph 97:

When considering the data presented here as a whole, the superiority of the PMO targeting the sequence +30+59 (PMO-G, or h53A30/1), is strongly indicated. In normal myoblasts, nucleofection of PMO-G (300 nM) and liposomal-carrier mediated transfection of

leashed PMO-G (500 nM) produced over 80% and over 50% skipping of exon 53, respectively, implying that it acts extremely efficiently within the cell. This was confirmed in patient cells. Indeed, this PMO generates the highest levels of exon skipping in patient cells over a range of concentrations (up to 200 nM) and, most important therapeutically, exerts its activity at concentrations as low as 25 nM. The exon skipping activity of this PMO is also persistent, with over 70% exon skipping for 7 days in culture, and over 60% exon skipping for up to three weeks. This would have important safety and cost implications as a genetic therapy for DMD patients with the appropriate deletions. PMO-G was also shown to skip exon 53 correctly in vivo. These RNA results were further confirmed by the detection of dystrophin protein at a high level in protein extracts from patient cells treated with PMO-G. Previous studies by the Leiden group [18] suggest that the optimal 2'OMePS AO is targeted to the sequence +46+63 of exon 53, producing exon skipping in up to 25% of transcripts in cultured cells and 7% in the hDMD mouse. This 2'OMePS AO shows some degree of overlap with the optimal PMOs reported here which strengthens our findings. The reason that our optimal PMO is more specific could be a (combined) consequence of the different AO chemistries, length of AO used, and the absolute target site of AO.

The prior art has therefore taught that the same region targeted by the instantly claimed oligomers is superior to other regions of exon 53. The prior art has taught that sequences with SEQ ID NOS:10-12 are included in their invention. The recited SEQ ID NOS: fall squarely within SEQ ID NOS:10-12 and 24 which has been taught by Popplewell to be a “superior” target region of exon 53. While the entire document is pertinent to applicant invention, please also see Example 2 and claims 1-12.

Sazani et al have also taught antisense oligomers for inducing exon 53 skipping in the human dystrophin gene. Sazani et al have also taught oligomers targeting the same target site and the instant invention and the superior region taught by Popplewell et all See for example SEQ ID NOS: 430, 431, and 628-633 which all overlap or is/are embrace the instantly recited SEQ ID NOS. Sazani et al have also taught that oligomer size choices and modification of antisense oligonucleotides. while the entire reference is

relied upon and relevant to applicants invention, applicant is directed to, for example, paragraphs 18-25, 36, 40, 50, 56, 95, 97, 98, 104, 118, 123-177, 196, 197, and claims 36-39, for example.

While the prior art has not specifically disclosed the recited sequences SEQ ID Nos, the prior art has clearly taught that such sequences are embraced within a known target region and furthermore within known antisense compounds. The prior art, however, has taught that the region that the instant compounds are targeted to is a superior target region and furthermore the prior art references taken together have taught that one in the art can alter the sizes of the antisense compounds. It would have been well within the skill of the artisan to test various sizes of oligomers in an optimization of antisense compounds targeting a known superior target region. It is noted that the superior target region is not large; "When considering the data presented here as a whole, the superiority of the PMO targeting the sequence +30+59 (PMO-G, or h53A30/1), is strongly indicated." Applicants invention is oligomers that are within this exemplified compound where it has been clearly taught that sequences within this oligomer were considered y by the prior art. The modifications utilized in the invention and recited in the claims were well known and routinely used in the art at the time of invention as shown by the above art and evidenced by Baker et al and Bennett et al. The benefits of the modifications were well known in the art where nuclease protection, and improved hybridization, and cell penetration were known benefits, for example. Both of these references are drawn to antisense compounds utilized in alteration of splicing. See

Paragraphs 10, 11, 13, 27, and 60-71 of Baker et al and paragraphs 25, 57-75, 97-104, 140-155, 176, and 180-183 of Bennett et al, for example. Bennett and Baker et al have also taught various size ranges for splice altering antisense compounds.

The invention as a whole would therefore have been *prima facie* obvious to one in the art at the time of invention.

Response to Arguments

Applicant's arguments filed 7/22/2016 have been fully considered but they are not persuasive.

Applicant has argued that the rejection of record fails to articulate why a skilled artsen would have been guided or directed to modify the antisense oligomers of Sazani to arrive at the presently claimed oligomers. It is noted that the instant oligomers are targeted to nt32-56 and 36-60 of exon 53. These oligomers are 25mers. The examiner asserts that the rejection specifically provides the ese teachings from the prior art.

From the rejection above:

It has been taught to target exon 53 to induce skipping of the 53rd exon. The specific sequences and modifications recited in the instant claims have been clearly suggested by Popplewell et al. See for example SEQ ID NOS:10-12 and 24, and paragraph 15:

The molecule that causes skipping in exon 53 comprises at least a 25 base length from a base sequence selected from:

TABLE-US-00005 (SEQ ID NO: 10) j) CXG XXG CCX CCG GXX CXG AAG GXG XXC XXG; (SEQ ID NO: 11) k) CAA CXG XXG CCX CCG GXX CXG AAG GXG XXC; or (SEQ ID NO: 12) l) XXG CCX CCG GXX CXG AAG GXG XXC XXG XAC,

wherein the molecule's sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in exon 53 of the dystrophin gene.

Paragraph 32:

The molecule is at least 25 bases in length. Preferably, the molecule is at least 28 bases in length. Preferably, the molecule is no more than 35 bases in length and, more preferably, no more than 32 bases in length. Preferably, the molecule is between 25 and 35 bases in length, more preferably, the molecule is between 28 and 32 bases in length, even more preferably, the molecule is between 29 and 31 bases in length, and most preferably, the molecule is 30 bases in length. It has been found that a molecule which is 30 bases in length causes efficient exon skipping. If the molecule is longer than 35 bases in length, the specificity of the binding to the specific exon region is reduced. If the molecule is less than 25 bases in length, the exon skipping efficiency is reduced.

Paragraph 97:

When considering the data presented here as a whole, the superiority of the PMO targeting the sequence +30+59 (PMO-G, or h53A30/1), is strongly indicated. In normal myoblasts, nucleofection of PMO-G (300 nM) and liposomal-carrier mediated transfection of leashed PMO-G (500 nM) produced over 80% and over 50% skipping of exon 53, respectively, implying that it acts extremely efficiently within the cell. This was confirmed in patient cells. Indeed, this PMO generates the highest levels of exon skipping in patient cells over a range of concentrations (up to 200 nM) and, most important therapeutically, exerts its activity at concentrations as low as 25 nM. The exon skipping activity of this PMO is also persistent, with over 70% exon skipping for 7 days in culture, and over 60% exon skipping for up to three weeks. This would have important safety and cost implications as a genetic therapy for DMD patients with the appropriate deletions. PMO-G was also shown to skip exon 53 correctly in vivo. These RNA results were further confirmed by the detection of dystrophin protein at a high level in protein extracts from patient cells treated with PMO-G. Previous studies by the Leiden group [18] suggest that the optimal 2'OMePS AO is targeted to the sequence +46+63 of exon 53, producing exon skipping in up to 25% of transcripts in cultured cells and 7% in the hDMD mouse. This 2'OMePS AO shows some degree of overlap with the optimal PMOs reported here which strengthens our findings. The reason that our optimal PMO is more specific could be a (combined) consequence of the different AO chemistries, length of AO used, and the absolute target site of AO.

The prior art has therefore taught that the same region targeted by the instantly claimed oligomers is superior to other regions of exon 53. The prior art has taught that sequences with SEQ ID NOS:10-12 are included in their invention. The recited SEQ ID NOS: fall squarely within SEQ ID NOS:10-12 and 24 which has been taught by Popplewell to be a “superior” target region of exon 53. While the entire document is pertinent to applicant invention, please also see Example 2 and claims 1-12.

Applicant dismissed the Baker and Bennet references since they are not directed to the same gene. This is not what the references are relied on for. For example they are relied on for what was asserted in the rejection above: modifications of antisense compounds utilized for intron splice modulation.

Applicant asserts that the instant compounds have unexpected properties. The examiner disagrees. The compounds function as designed, to alter splicing. The fact that applicant screened for more oligonucleotides in a region that has been taught to be superior utilizing size ranges and modifications known in the art is not unexpected. The prior art indeed asserts that these oligonucleotides are included in their invention as asserted in the rejection of record.

Double Patenting

Claims 1 and 15-25 **WERE** rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-7 of U.S. Patent No. 9079934.

This rejection has been withdrawn in view if the amendments filed 7/22/2016.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to SEAN MCGARRY whose telephone number is (571)272-0761. The examiner can normally be reached on M-Th (7:00-5:30).

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Shaojia Anna Jiang can be reached on (571) 272-0627. The fax phone

number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

Sean R McGarry
Primary Examiner
Art Unit 1674

/SEAN MCGARRY/
Primary Examiner, Art Unit 1674

Docket No.: 209658-0001-01-US-518587
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Naoki WATANABE et al.

Application No.: 14/615,504 Confirmation No.: 2704

Filed: February 6, 2015 Art Unit: 1674

For: ANTISENSE NUCLEIC ACIDS Examiner: S. McGarry

AMENDMENT / RESPONSE UNDER 37 C.F.R. § 1.116
&
PETITION FOR EXTENSION OF TIME

MS AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the FINAL Office Action mailed October 27, 2016, the Office is respectfully requested to consider and enter the following amendments and remarks. Applicant petitions herewith a ONE-month extension of time, extending the period of response until February 27, 2017.

Amendments to the Claims begin on page 2.

Remarks begin on page 4.

A **Certification and Request for Consideration under the After Final Consideration Pilot Program 2.0** is concurrently submitted.

87880460.1

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions, and listings, of the claims in the application:

LISTING OF CLAIMS

Claim 1. (Currently Amended): An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of the nucleotide sequence of ~~SEQ ID NO: 11 and~~ SEQ ID NO: 57, wherein the antisense oligomer is an oligonucleotide in which the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified, or a morpholino oligomer.

Claims 2-16. (Canceled).

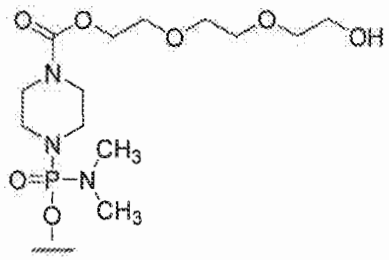
Claim 17. (Previously Presented): The antisense oligomer according to claim 1, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of: OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br, and I, wherein R is an alkyl or an aryl and R' is an alkylene.

Claim 18. (Previously Presented): The antisense oligomer according to claim 1, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of: a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond, and a boranophosphate bond.

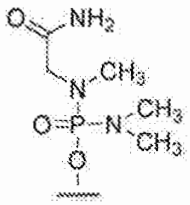
Claim 19. (Previously Presented): The antisense oligomer according to claim 1, which is a morpholino oligomer.

Claim 20. (Previously Presented): The antisense oligomer according to claim 19, which is a phosphorodiamidate morpholino oligomer.

Claim 21. (Previously Presented): The antisense oligomer according to claim 19, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:



(1)



(2)

(3)

Claims 22-24. (Canceled).

Claim 25. (Previously Presented): A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to claim 1, or a pharmaceutically acceptable salt or hydrate thereof.

REMARKS

Entry of this Amendment is proper under 37 C.F.R. § 1.116, because the Amendment places the application in condition for allowance for the reasons discussed herein; does not raise any new issue requiring further search and/or consideration, because the amendments amplify issues previously discussed throughout prosecution; relates to matters of form rather than substance, because the added language was already present in the claims and thus presents no additional search burden; adds no new claims; and places the application in a better form for an appeal should an appeal be necessary. The Amendment is necessary and was not earlier presented because it is made in response to arguments raised in the final rejection. Entry of the Amendment, reexamination, and further and favorable reconsideration of the subject application given the following remarks, pursuant to and consistent with 37 C.F.R. § 1.116, are thus respectfully requested.

1. Status of the Claims and Support for the Claim Amendments

The status of the claims following entry of the amendments is as follows:

Claims pending: 1, 17-21, and 25

Claims rejected: 1, 17-21, and 23-25

Claim amended: 1

Claims canceled: 2-16 and 22-24

Applicants amend claim 1 to no longer recite SEQ ID NO: 11. Thus, no prohibited new matter is believed to be added.

The claims have been amended without prejudice to, or disclaimer of, the canceled subject matter. Applicants reserve the right to file a continuation or divisional application on any subject matter canceled by way of amendments.

2. Status the Drawings

Applicants appreciate the Office's acknowledgment that the drawings filed February 6, 2015 and March 3, 2015 are accepted by the Office.

3. Priority Documents

Applicants appreciates the Office's acknowledgment that certified copies of the priority document have been received in the parent application.

4. Withdrawn Objections and Rejections

Rejections and objections not reiterated stand withdrawn. *See* 37 C.F.R. § 1.113(b); M.P.E.P. §§ 706.07 and 707.07(e).

5. Claim Rejection under 35 U.S.C. § 103(a)

The Office rejects claims 1, 17-21, and 23-25 as allegedly obvious over **Popplewell** et al., U.S. Published Patent Application No. 2010/0168212 ("Popplewell") and **Sazani** et al., U.S. Published Patent Application No. 2010/0130591 ("Sazani") in view of **Baker** et al., U.S. Published Patent Application No. 2013/0109091 ("Baker") and **Bennett** et al., U.S. Published Patent Application No. 2012/0190728 ("Bennett"). Office Action, pages 2-10.

Alleged Grounds for Rejection

Popplewell allegedly teaches targeting the 53rd exon of the human dystrophin gene to induce skipping of the 53rd exon. *Id.*, at 4. Popplewell's SEQ ID NOs: 10-12 and 24 allegedly suggest the presently recited sequences and modifications. *Id.*, 4 and 8. Sazani allegedly teaches antisense oligomers for inducing exon 53 skipping in the human dystrophin gene. *Id.*, at 6-7. Sazani's SEQ ID NOs: 430-431 and 628-633 allegedly overlap or encompass the presently recited SEQ ID NOs. *Id.* The Office admits that none of the cited references discloses the presently recited SEQ ID NOs. *Id.*, at 8. Nevertheless, the Office alleges that "[i]t would have been well within the skill of the artisan to test various sizes of oligomers in an optimization of antisense compounds targeting a known superior target region." *Id.*

The Office by relying upon Baker and Bennett further alleges that "[t]he modifications utilized in the invention and recited in the claims were well known and routinely used in the art at the time." *Id.*, 7. The Office then concludes that "[t]he invention as whole would therefore have been prima facie obvious to one in the art at the time of invention." *Id.*, 8.

Given Applicants' Amendment/Response filed July 22, 2016, the Office discounts Applicants' argument. *Id.*, 10. The presently recited SEQ ID Nos "falls squarely within" what "has been taught by Popplewell to be a 'superior' target region." *Id.* The Office further alleges that "[t]he prior art indeed asserts that these oligonucleotides are included in their invention as asserted in the rejection of record." *Id.*

Arguments

Applicants traverse the rejection to the extent it may be applied to the amended claims. Both the suggestion of the claimed invention and the expectation of success must be in the prior art, not in the disclosure of the claimed invention. *In re Dow Chem. Co.*, 837 F.2d 469 (Fed. Cir. 1988). Additionally, once the scope and content of the prior art are determined, the relevant inquiry is whether the prior art suggests all the limitations of the invention, and whether one of ordinary skill in the art would have had a reasonable expectation that the claimed invention would be successful as so combined. *In re Vaeck*, 947 F.2d 488, 493 (Fed. Cir. 1991).

Upon entry of the present amendments, independent claim 1 recites, *inter alia*, an oligomer consisting of the nucleotide sequence of SEQ ID NO: 57. As the Office admits, none of the cited references teaches or suggests SEQ ID NO: 57. The Office's rejection is unsupported, at least because the Office fails to articulate a rationale why a skilled artisan would have been guided or directed to modify the antisense oligomers of the cited references to arrive at the presently claimed antisense oligomers. Without such guidance, the artisan would not have had a reasonable expectation of success in arriving at the claimed sequences. *See, e.g., In re O'Farrell*, 853 F.2d 894, 903 (Fed. Cir. 1988).[1] Teachings of Baker and Bennett are not directly applicable, because the targeted genes discussed therein differ from the human dystrophin gene.

In fact, a skilled artisan given Popplewell would have been directed to use or modify the oligomers that are different from the presently recited ones. Popplewell teaches that the oligomer corresponding to positions 30-59 of exon 53 provides the highest activity. *See, e.g.,*

[1] "The admonition that 'obvious to try' is not the standard under § 103 has been directed mainly at two kinds of error. In some cases, what would have been 'obvious to try' would have been to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful."

Popplewell, ¶ [0074][2] and FIG. 8. In contrast, the presently recited SEQ ID NO: 57 corresponds to positions 36-60 of exon 50. Thus, Popplewell's top performer is different from the presently recited ones. There is no evidence on the record, or adduced by the Office, that a skilled artisan given Popplewell's teachings would have arrived at the presently recited oligomers, let alone doing so with any expectation of success.

Additionally, the presently recited oligomer (consisting of the nucleotide sequence of SEQ ID NO: 57) offer superior skipping effects over the oligomers taught in both Popplewell and Sazani. For example, Figures 2-4 of the Specification (corresponding to data in Test Examples 2-3) show that PMO No. 3 (having the nucleotide sequence of SEQ ID NO: 11; *see* Table 2) outperformed exemplary antisense oligomers taught in Popplewell (PMO Nos. 12 and 15). As shown in TABLE 2, PMO Nos. 12 and 15 corresponds to the top performer taught in Popplewell (targeting sequence 30-59 of exon 53). Additionally, Figures 18-19 of the Specification (corresponding to data in Test Example 7) show that PMO No. 3 (having the nucleotide sequence of SEQ ID NO: 11; *see* Table 2) outperformed exemplary antisense oligomer taught in Sazani (PMO No. 16).[3] Furthermore, Figures 16-17 (corresponding to data in Test Example 6) show that the oligomer having the nucleotide sequence of SEQ ID NO: 57 (H53_36-60) displays a higher level skipping activity that that having the nucleotide sequence of SEQ ID NO: 11 (H53_32-56). Thus, the recited oligomers consisting of the nucleotide sequence of SEQ ID NO: 57 also have superior skipping activity over exemplary oligomers taught in Popplewell and Sazani, particularly the top performer taught in Popplewell. Applicants submit that this superiority is unexpected, at least because none of the cited references teach or suggest such an effect.

Given at least the above arguments, claim 1 as amended and its dependent claims 17-21, and 25 would have been nonobvious over cited references. Claims 23-24 stand canceled,

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[2] "…produced the most robust skipping of exon 53, and should be considered the sequence of choice for any upcoming PMO clinical trial."

[3] Figure 19 shows that PMO No. 3 has an equivalent level of skipping activity as PMO No. 8. Figure 18 shows that PMO No. 8 has a higher level of skipping activity than the exemplary antisense oligomer taught in Sazani (PMO No. 16). Thus, a skilled artisan given Figures 18-19 would have understood the following order of the skipping activities:

PMO No. 3 ≈ PMO No. 8 >> PMO No. 16.

mooting at least this aspect of the rejection. Accordingly, Applicants respectfully request withdrawal of the rejection and allowance of the claims.

CONCLUSION

In view of the foregoing, Applicants submit that the pending claims are in condition for allowance, and respectfully request reconsideration and timely allowance of the pending claims. Should the Office have any questions or comments regarding Applicants' amendments or response, please contact Applicants' undersigned representative at (202) 230-5119. Furthermore, please direct all correspondence to the below-listed address.

EXCEPT for issue fees payable under 37 C.F.R. § 1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. § 1.16 and 1.17 which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account No. 50-0573. If an Appeal fee is required to maintain pendency of the present application, the Office is authorized to charge the Appeal fee to the deposit account above and use this paper as a constructive Notice of Appeal.

Dated: February 27, 2017

Respectfully submitted,

Customer Number: 055694

By _____
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Attorneys/Agents For Applicant

87880460.1

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
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NOTICE OF ALLOWANCE AND FEE(S) DUE

55694 7590 03/10/2017
DRINKER BIDDLE & REATH (DC)
1500 K STREET, N.W.
SUITE 1100
WASHINGTON, DC 20005-1209

EXAMINER
MCGARRY, SEAN

ART UNIT	PAPER NUMBER
1674	

DATE MAILED: 03/10/2017

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/615,504	02/06/2015	Naoki WATANABE	209658-0001-01-US-518587	2704

TITLE OF INVENTION: ANTISENSE NUCLEIC ACIDS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$0	$960	06/12/2017

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

55694 7590 03/10/2017
DRINKER BIDDLE & REATH (DC)
1500 K STREET, N.W.
SUITE 1100
WASHINGTON, DC 20005-1209

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission
I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/615,504	02/06/2015	Naoki WATANABE	209658-0001-01-US-518587	2704

TITLE OF INVENTION: ANTISENSE NUCLEIC ACIDS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$960	$0	$0	$960	06/12/2017

EXAMINER	ART UNIT	CLASS-SUBCLASS
MCGARRY, SEAN	1674	536-024500

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
- ❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- ❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.
1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. The following fee(s) are submitted:
- ❑ Issue Fee
- ❑ Publication Fee (No small entity discount permitted)
- ❑ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**
- ❑ A check is enclosed.
- ❑ Payment by credit card. Form PTO-2038 is attached.
- ❑ The director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
- ❑ Applicant certifying micro entity status. See 37 CFR 1.29
  NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
- ❑ Applicant asserting small entity status. See 37 CFR 1.27
  NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
- ❑ Applicant changing to regular undiscounted fee status.
  NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

PTOL-85 Part B (10-13) Approved for use through 10/31/2013. OMB 0651-0033 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/615,504	02/06/2015	Naoki WATANABE	209658-0001-01-US-518587	2704

55694 7590 03/10/2017

DRINKER BIDDLE & REATH (DC)
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WASHINGTON, DC 20005-1209

EXAMINER	
MCGARRY, SEAN	

ART UNIT	PAPER NUMBER
1674	

DATE MAILED: 03/10/2017

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 14/615,504	Applicant(s) WATANABE ET AL.	
	Examiner SEAN MCGARRY	Art Unit 1674	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *AF filed 2/27/2017*.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on_____.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are *7,17-21 and 25*. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.
4. ☒ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☒ All b) ☐ Some *c) ☐ None of the:
   1. ☐ Certified copies of the priority documents have been received.
   2. ☒ Certified copies of the priority documents have been received in Application No. *13/819,520* .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
5. ☐ Examiner's Amendment/Comment
6. ☐ Examiner's Statement of Reasons for Allowance
7. ☒ Other *PTO-2323*.

/SEAN MCGARRY/
Primary Examiner, Art Unit 1674

AFCP 2.0 Decision	Application No.	Applicant(s)
	14/615,504	WATANABE ET AL.
	Examiner	Art Unit
	SEAN MCGARRY	1674

This is in response to the After Final Consideration Pilot request filed 2/27/2017.

1. **Improper Request** – The AFCP 2.0 request is improper for the following reason(s) and the after final amendment submitted with the request will be treated under pre-pilot procedure.

☐ An AFCP 2.0 request form PTO/SB/434 (or equivalent document) was not submitted.

☐ A non-broadening amendment to at least one independent claim was not submitted.

☐ A proper AFCP 2.0 request was submitted in response to the most recent final rejection.

☐ Other:

2. **Proper Request**

A. After final amendment submitted with the request will not be treated under AFCP 2.0.
The after final amendment cannot be reviewed and a search conducted within the guidelines of the pilot program.

☐ The after final amendment will be treated under pre-pilot procedure.

B. Updated search and/or completed additional consideration.
The examiner performed an updated search and/or completed additional consideration of the after final amendment within the time authorized for the pilot program. The result(s) of the updated search and/or completed additional consideration are:

☒ 1. All of the rejections in the most recent final Office action are overcome and a Notice of Allowance is issued herewith.

☐ 2. The after final amendment would not overcome all of the rejections in the most recent final Office action. See attached interview summary for further details.

☐ 3. The after final amendment was reviewed, and it raises a new issue(s). See attached interview summary for further details.

☐ 4. The after final amendment raises new issues, but would overcome all of the rejections in the most recent final Office action. A decision on determining allowability could not be made within the guidelines of the pilot. See attached interview summary for further details, including any newly discovered prior art.

☐ 5. Other:

Examiner Note: Please attach an interview summary when necessary as described above.

EXHIBIT 4

MOLECULAR BIOLOGY OF

THE CELL

ALBERTS JOHNSON LEWIS RAFF ROBERTS WALTER

FOURTH EDITION

MOLECULAR BIOLOGY OF THE CELL

fourth edition

Bruce Alberts

Alexander Johnson

Julian Lewis

Martin Raff

Keith Roberts

Peter Walter

Garland Science
Taylor & Francis Group

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Cell Biology Interactive
Artistic and Scientific Direction: Peter Walter
Narrated by: Julie Theriot
Production, Design, and Development: Mike Morales

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Front cover Human Genome: Reprinted by permission from *Nature,* International Human Genome Sequencing Consortium, 409:860–921, 2001 © Macmillan Magazines Ltd. Adapted from an image by Francis Collins, NHGRI; Jim Kent, UCSC; Ewan Birney, EBI; and Darryl Leja, NHGRI; showing a portion of Chromosome 1 from the initial sequencing of the human genome.

Chapter opener Portion of chromosome 2 from the genome of the fruit fly *Drosophila melanogaster.* (Reprinted with permission from M.D. Adams et al., *Science* 287:2185–2195, 2000. © AAAS.)

Back cover In 1967, the British artist Peter Blake created a design classic. Nearly 35 years later Nigel Orme (illustrator), Richard Denyer (photographer), and the authors have together produced an affectionate tribute to Mr Blake's image. With its gallery of icons and influences, its assembly created almost as much complexity, intrigue and mystery as the original. *Drosophila, Arabidopsis,* Dolly and the assembled company tempt you to dip inside where, as in the original, "a splendid time is guaranteed for all."
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Contents

4

DNA AND CHROMOSOMES

Life depends on the ability of cells to store, retrieve, and translate the genetic instructions required to make and maintain a living organism. This *hereditary* information is passed on from a cell to its daughter cells at cell division, and from one generation of an organism to the next through the organism's reproductive cells. These instructions are stored within every living cell as its **genes**, the information-containing elements that determine the characteristics of a species as a whole and of the individuals within it.

As soon as genetics emerged as a science at the beginning of the twentieth century, scientists became intrigued by the chemical structure of genes. The information in genes is copied and transmitted from cell to daughter cell millions of times during the life of a multicellular organism, and it survives the process essentially unchanged. What form of molecule could be capable of such accurate and almost unlimited replication and also be able to direct the development of an organism and the daily life of a cell? What kind of instructions does the genetic information contain? How are these instructions physically organized so that the enormous amount of information required for the development and maintenance of even the simplest organism can be contained within the tiny space of a cell?

The answers to some of these questions began to emerge in the 1940s, when researchers discovered, from studies in simple fungi, that genetic information consists primarily of instructions for making proteins. Proteins are the macromolecules that perform most cellular functions: they serve as building blocks for cellular structures and form the enzymes that catalyze all of the cell's chemical reactions (Chapter 3), they regulate gene expression (Chapter 7), and they enable cells to move (Chapter 16) and to communicate with each other (Chapter 15). The properties and functions of a cell are determined almost entirely by the proteins it is able to make. With hindsight, it is hard to imagine what other type of instructions the genetic information could have contained.

The other crucial advance made in the 1940s was the identification of **deoxyribonucleic acid (DNA)** as the likely carrier of genetic information. But the mechanism whereby the hereditary information is copied for transmission from cell to cell, and how proteins are specified by the instructions in the DNA, remained completely mysterious. Suddenly, in 1953, the mystery was solved when the structure of DNA was determined by James Watson and Francis Crick. As mentioned in Chapter 1, the structure of DNA immediately solved the problem of how the information in this molecule might be copied, or *replicated*. It also provided the first clues as to how a molecule of DNA might encode the instructions for making proteins. Today, the fact that DNA is the genetic material is so fundamental to biological thought that it is difficult to realize what an enormous intellectual gap this discovery filled.

Well before biologists understood the structure of DNA, they had recognized that genes are carried on *chromosomes*, which were discovered in the nineteenth century as threadlike structures in the nucleus of a eucaryotic cell that become visible as the cell begins to divide (Figure 4–1). Later, as biochemical analysis became possible, chromosomes were found to consist of both DNA and protein. We now know that the DNA carries the hereditary information of the cell (Figure 4–2). In contrast, the protein components of chromosomes function largely to package and control the enormously long DNA molecules so that they fit inside cells and can easily be accessed by them.

In this chapter we begin by describing the structure of DNA. We see how, despite its chemical simplicity, the structure and chemical properties of DNA make it ideally suited as the raw material of genes. The genes of every cell on Earth are made of DNA, and insights into the relationship between DNA and genes have come from experiments in a wide variety of organisms. We then consider how genes and other important segments of DNA are arranged on the long molecules of DNA that are present in chromosomes. Finally, we discuss how eucaryotic cells fold these long DNA molecules into compact chromosomes. This packing has to be done in an orderly fashion so that the chromosomes can be replicated and apportioned correctly between the two daughter cells at each cell division. It must also allow access of chromosomal DNA to enzymes that repair it when it is damaged and to the specialized proteins that direct the expression of its many genes.

This is the first of four chapters that deal with basic genetic mechanisms—the ways in which the cell maintains, replicates, expresses, and occasionally improves the genetic information carried in its DNA. In the following chapter (Chapter 5) we discuss the mechanisms by which the cell accurately replicates and repairs DNA; we also describe how DNA sequences can be rearranged through the process of genetic recombination. Gene expression—the process through which the information encoded in DNA is interpreted by the cell to guide the synthesis of proteins—is the main topic of Chapter 6. In Chapter 7, we describe how gene expression is controlled by the cell to ensure that each of the many thousands of proteins encrypted in its DNA is manufactured only at the proper time and place in the life of the cell. Following these four chapters on basic genetic mechanisms, we present an account of the experimental techniques used to study these and other processes that are fundamental to all cells (Chapter 8).

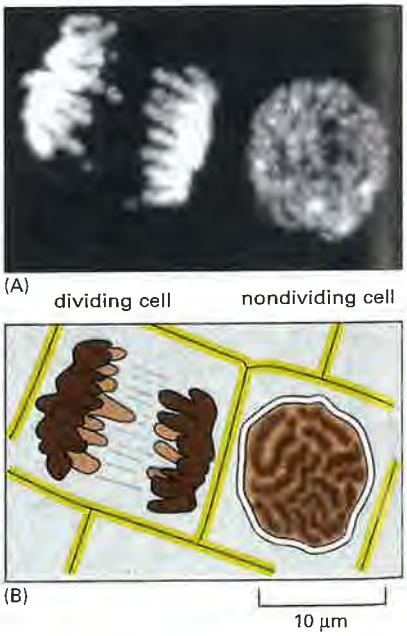

Figure 4–1 Chromosomes in cells. (A) Two adjacent plant cells photographed through a light microscope. The DNA has been stained with a fluorescent dye (DAPI) that binds to it. The DNA is present in chromosomes, which become visible as distinct structures in the light microscope only when they become compact structures in preparation for cell division, as shown on the left. The cell on the right, which is not dividing, contains identical chromosomes, but they cannot be clearly distinguished in the light microscope at this phase in the cell's life cycle, because they are in a more extended conformation. (B) Schematic diagram of the outlines of the two cells along with their chromosomes. (A, courtesy of Peter Shaw.)

THE STRUCTURE AND FUNCTION OF DNA

Biologists in the 1940s had difficulty in accepting DNA as the genetic material because of the apparent simplicity of its chemistry. DNA was known to be a long polymer composed of only four types of subunits, which resemble one another chemically. Early in the 1950s, DNA was first examined by x-ray diffraction analysis, a technique for determining the three-dimensional atomic structure of a molecule (discussed in Chapter 8). The early x-ray diffraction results indicated that DNA was composed of two strands of the polymer wound into a helix. The observation that DNA was double-stranded was of crucial significance and

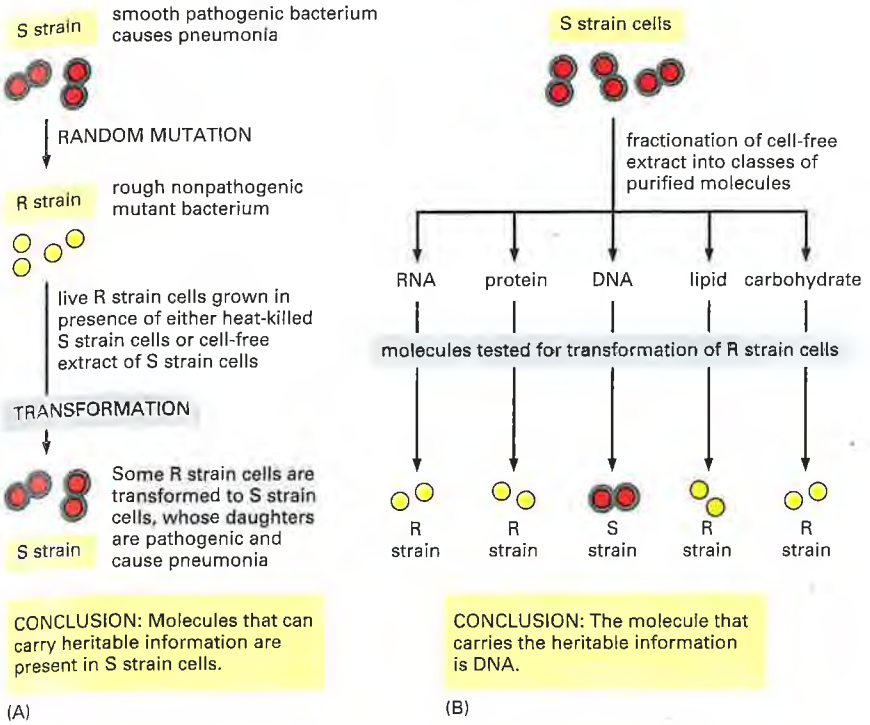

Figure 4–2 Experimental demonstration that DNA is the genetic material. These experiments, carried out in the 1940s, showed that adding purified DNA to a bacterium changed its properties and that this change was faithfully passed on to subsequent generations. Two closely related strains of the bacterium *Streptococcus pneumoniae* differ from each other in both their appearance under the microscope and their pathogenicity. One strain appears smooth (S) and causes death when injected into mice, and the other appears rough (R) and is nonlethal. (A) This experiment shows that a substance present in the S strain can change (or transform) the R strain into the S strain and that this change is inherited by subsequent generations of bacteria. (B) This experiment, in which the R strain has been incubated with various classes of biological molecules obtained from the S strain, identifies the substance as DNA.

provided one of the major clues that led to the Watson–Crick structure of DNA. Only when this model was proposed did DNA's potential for replication and information encoding become apparent. In this section we examine the structure of the DNA molecule and explain in general terms how it is able to store hereditary information.

A DNA Molecule Consists of Two Complementary Chains of Nucleotides

A DNA molecule consists of two long polynucleotide chains composed of four types of nucleotide subunits. Each of these chains is known as a *DNA chain*, or a *DNA strand*. *Hydrogen bonds* between the base portions of the nucleotides hold the two chains together (Figure 4–3). As we saw in Chapter 2 (Panel 2–6, pp. 120-121), nucleotides are composed of a five-carbon sugar to which are attached one or more phosphate groups and a nitrogen-containing base. In the case of the nucleotides in DNA, the sugar is deoxyribose attached to a single phosphate group (hence the name deoxyribonucleic acid), and the base may be either *adenine (A)*, *cytosine (C)*, *guanine (G)*, or *thymine (T)*. The nucleotides are covalently linked together in a chain through the sugars and phosphates, which thus form a "backbone" of alternating sugar–phosphate–sugar–phosphate (see Figure 4–3). Because only the base differs in each of the four types of subunits, each polynucleotide chain in DNA is analogous to a necklace (the backbone) strung with four types of beads (the four bases A, C, G, and T). These same symbols (A, C, G, and T) are also commonly used to denote the four different nucleotides—that is, the bases with their attached sugar and phosphate groups.

The way in which the nucleotide subunits are lined together gives a DNA strand a chemical polarity. If we think of each sugar as a block with a protruding knob (the 5′ phosphate) on one side and a hole (the 3′ hydroxyl) on the other (see Figure 4–3), each completed chain, formed by interlocking knobs with holes, will have all of its subunits lined up in the same orientation. Moreover, the two ends of the chain will be easily distinguishable, as one has a hole (the 3′ hydroxyl) and the other a knob (the 5′ phosphate) at its terminus. This polarity in a DNA chain is indicated by referring to one end as the *3′ end* and the other as the *5′ end*.

The three-dimensional structure of DNA—**the double helix**—arises from the chemical and structural features of its two polynucleotide chains. Because

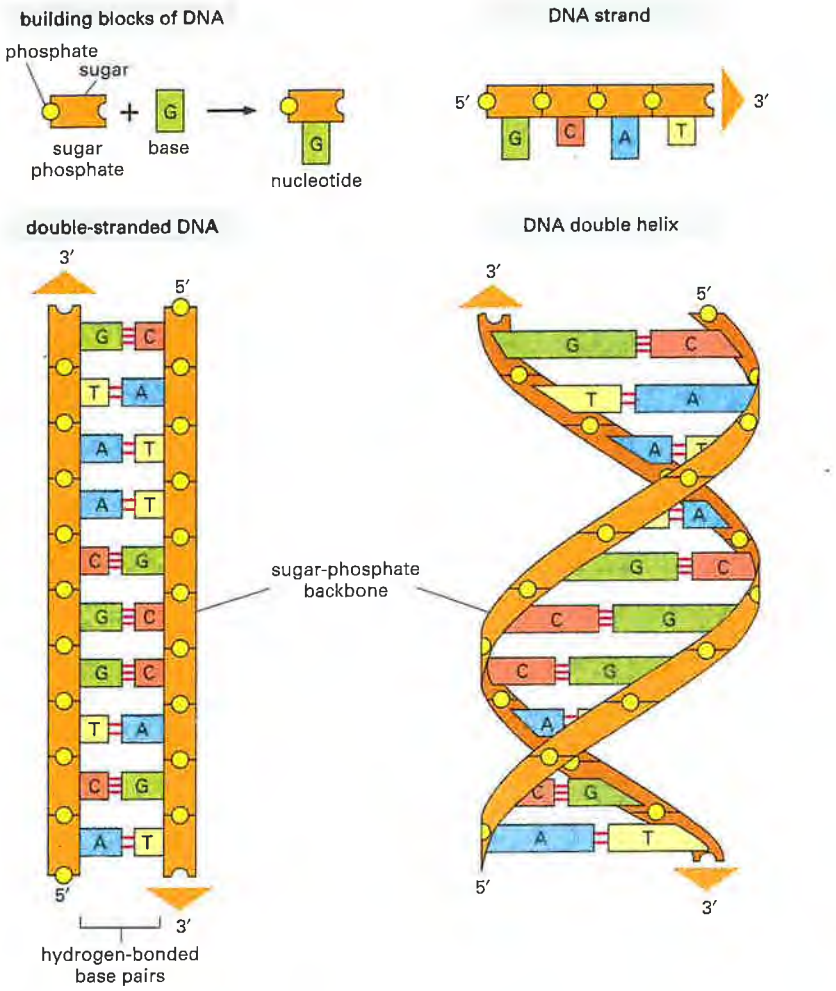

Figure 4–3 DNA and its building blocks. DNA is made of four types of nucleotides, which are linked covalently into a polynucleotide chain (a DNA strand) with a sugar-phosphate backbone from which the bases (A, C, G, and T) extend. A DNA molecule is composed of two DNA strands held together by hydrogen bonds between the paired bases. The *arrowheads* at the ends of the DNA strands indicate the polarities of the two strands, which run antiparallel to each other in the DNA molecule. In the diagram at the bottom left of the figure, the DNA molecule is shown straightened out; in reality, it is twisted into a double helix, as shown on the right. For details, see Figure 4–5.

these two chains are held together by hydrogen bonding between the bases on the different strands, all the bases are on the inside of the double helix, and the sugar-phosphate backbones are on the outside (see Figure 4–3). In each case, a bulkier two-ring base (a purine; see Panel 2–6, pp. 120–121) is paired with a single-ring base (a pyrimidine); A always pairs with T, and G with C (Figure 4–4). This *complementary base-pairing* enables the **base pairs** to be packed in the

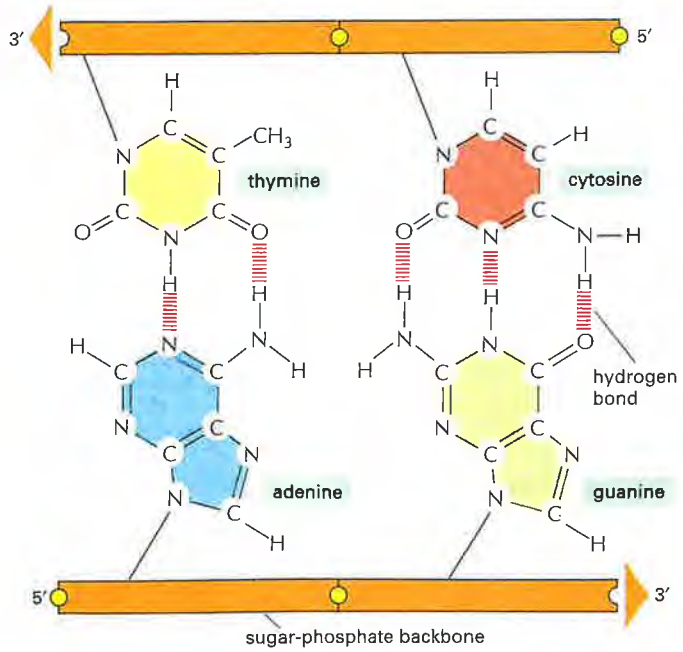

Figure 4–4 Complementary base pairs in the DNA double helix. The shapes and chemical structure of the bases allow hydrogen bonds to form efficiently only between A and T and between G and C, where atoms that are able to form hydrogen bonds (see Panel 2–3, pp. 114–115) can be brought close together without distorting the double helix. As indicated, two hydrogen bonds form between A and T, while three form between G and C. The bases can pair in this way only if the two polynucleotide chains that contain them are antiparallel to each other.

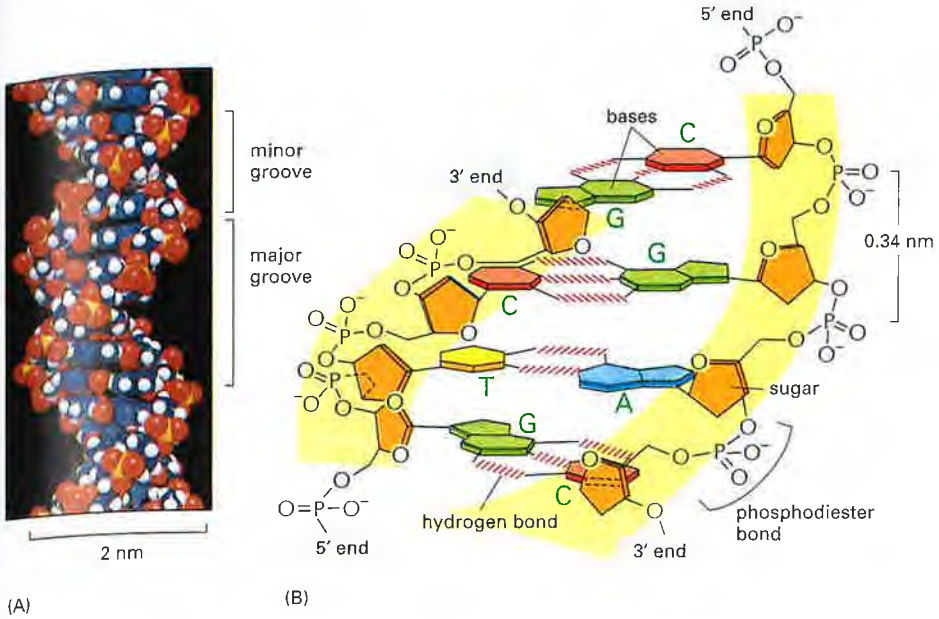

Figure 4–5 The DNA double helix. (A) A space-filling model of 1.5 turns of the DNA double helix. Each turn of DNA is made up of 10.4 nucleotide pairs and the center-to-center distance between adjacent nucleotide pairs is 3.4 nm. The coiling of the two strands around each other creates two grooves in the double helix. As indicated in the figure, the wider groove is called the major groove, and the smaller the minor groove. (B) A short section of the double helix viewed from its side, showing four base pairs. The nucleotides are linked together covalently by phosphodiester bonds through the 3′-hydroxyl (–OH) group of one sugar and the 5′-phosphate (P) of the next. Thus, each polynucleotide strand has a chemical polarity; that is, its two ends are chemically different. The 3′ end carries an unlinked –OH group attached to the 3′ position on the sugar ring; the 5′ end carries a free phosphate group attached to the 5′ position on the sugar ring.

energetically most favorable arrangement in the interior of the double helix. In this arrangement, each base pair is of similar width, thus holding the sugar-phosphate backbones an equal distance apart along the DNA molecule. To maximize the efficiency of base-pair packing, the two sugar-phosphate backbones wind around each other to form a double helix, with one complete turn every ten base pairs (Figure 4–5).

The members of each base pair can fit together within the double helix only if the two strands of the helix are **antiparallel**—that is, only if the polarity of one strand is oriented opposite to that of the other strand (see Figures 4–3 and 4–4). A consequence of these base-pairing requirements is that each strand of a DNA molecule contains a sequence of nucleotides that is exactly **complementary** to the nucleotide sequence of its partner strand.

The Structure of DNA Provides a Mechanism for Heredity

Genes carry biological information that must be copied accurately for transmission to the next generation each time a cell divides to form two daughter cells. Two central biological questions arise from these requirements: how can the information for specifying an organism be carried in chemical form, and how is it accurately copied? The discovery of the structure of the DNA double helix was a landmark in twentieth-century biology because it immediately suggested answers to both questions, thereby resolving at the molecular level the problem of heredity. We discuss briefly the answers to these questions in this section, and we shall examine them in more detail in subsequent chapters.

DNA encodes information through the order, or sequence, of the nucleotides along each strand. Each base—A, C, T, or G—can be considered as a letter in a four-letter alphabet that spells out biological messages in the chemical structure of the DNA. As we saw in Chapter 1, organisms differ from one another because their respective DNA molecules have different nucleotide sequences and, consequently, carry different biological messages. But how is the nucleotide alphabet used to make messages, and what do they spell out?

As discussed above, it was known well before the structure of DNA was determined that genes contain the instructions for producing proteins. The DNA messages must therefore somehow encode proteins (Figure 4–6). This relationship immediately makes the problem easier to understand, because of the chemical character of proteins. As discussed in Chapter 3, the properties of a protein, which are responsible for its biological function, are determined by its three-dimensional structure, and its structure is determined in turn by the linear

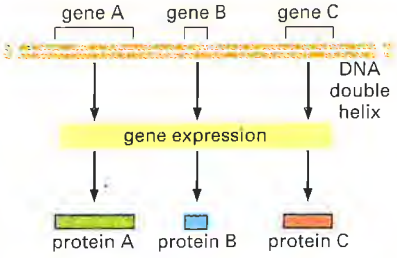

Figure 4–6 The relationship between genetic information carried in DNA and proteins.

Figure 4–7 The nucleotide sequence of the human β-globin gene. This gene carries the information for the amino acid sequence of one of the two types of subunits of the hemoglobin molecule, which carries oxygen in the blood. A different gene, the α-globin gene, carries the information for the other type of hemoglobin subunit (a hemoglobin molecule has four subunits, two of each type). Only one of the two strands of the DNA double helix containing the β-globin gene is shown; the other strand has the exact complementary sequence. By convention, a nucleotide sequence is written from its 5′ end to its 3′ end, and it should be read from left to right in successive lines down the page as though it were normal English text. The DNA sequences highlighted in *yellow* show the three regions of the gene that specify the amino sequence for the β-globin protein. We see in Chapter 6 how the cell connects these three sequences together to synthesize a full-length β-globin protein.

CCCTGTGGAGCCACACCCTAGGGTTGGCCA
ATCTACTCCCAGGAGCAGGGAGGGCAGGAG
CCAGGGCTGGGCATAAAAGTCAGGGCAGAG
CCATCTATTGCTTACATTTGCTTCTGACAC
AACTGTGTTCACTAGCAACTCAAACAGACA
CCATGGTGCACCTGACTCCTGAGGAGAAGT
CTGCCGTTACTGCCCTGTGGGGCAAGGTGA
ACGTGGATGAAGTTGGTGGTGAGGCCCTGG
GCAGGTTGGTATCAAGGTTACAAGACAGGT
TTAAGGAGACCAATAGAAACTGGGCATGTG
GAGACAGAGAAGACTCTTGGGTTTCTGATA
GGCACTGACTCTCTCTGCCTATTGGTCTAT
TTTCCCACCCTTAGGCTGCTGGTGGTCTAC
CCTTGGACCCAGAGGTTCTTTGAGTCCTTT
GGGGATCTGTCCACTCCTGATGCTGTTATG
GGCAACCCTAAGGTGAAGGCTCATGGCAAG
AAAGTGCTCGGTGCCTTTAGTGATGGCCTG
GCTCACCTGGACAACCTCAAGGGCACCTTT
GCCACACTGAGTGAGCTGCACTGTGACAAG
CTGCACGTGGATCCTGAGAACTTCAGGGTG
AGTCTATGGGACCCTTGATGTTTTCTTTCC
CCTTCTTTTCTATGGTTAAGTTCATGTCAT
AGGAAGGGGAGAAGTAACAGGGTACAGTTT
AGAATGGGAAACAGACGAATGATTGCATCA
GTGTGGAAGTCTCAGGATCGTTTTAGTTTC
TTTTATTTGCTGTTCATAACAATTGTTTTC
TTTTGTTTAATTCTTGCTTTCTTTTTTTTT
CTTCTCCGCAATTTTTACTATTATACTTAA
TGCCTTAACATTGTGTATAACAAAAGGAAA
TATCTCTGAGATACATTAAGTAACTTAAAA
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TTTCAGGGCAATAATGATACAATGTATCAT
GCCTCTTTGCACCATTCTAAAGAATAACAG
TGATAATTTCTGGGTTAAGGCAATAGCAAT
ATTTCTGCATATAAATATTTCTGCATATAA
ATTGTAACTGATGTAAGAGGTTTCATATTG
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GCTAATCATGTTCATACCTCTTATCTTCCT
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TGTGCTGGCCCATCACTTTGGCAAAGAATT
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AGTGGTGGCTGGTGTGGCTAATGCCCTGGC
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TGTCCAATTTCTATTAAAGGTTCCTTTGTT
CCCTAAGTCCAACTACTAAACTGGGGGATA
TTATGAAGGGCCTTGAGCATCTGGATTCTG
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TGATGTATTTAAATTATTTCTGAATATTTT
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TTTAAAACATAAAGAAATGATGAGCTGTTC
AAACCTTGGCAAAATACACTATATCTTAAA
CTCCATGAAAGAAGGTGAGGCTGCAACCAG
CTAATGCACATTGGCAACAGCCCCTGATGC
CTATGCCTTATTCATCCCTCAGAAAAGGAT
TCTTGTAGAGGCTTGATTTGCAGGTTAAAG
TTTTGCTATGCTGTATTTTACATTACTTAT
TGTTTTAGCTGTCCTCATGAATGTCTTTTC

sequence of the amino acids of which it is composed. The linear sequence of nucleotides in a gene must therefore somehow spell out the linear sequence of amino acids in a protein. The exact correspondence between the four-letter nucleotide alphabet of DNA and the twenty-letter amino acid alphabet of proteins—the genetic code—is not obvious from the DNA structure, and it took over a decade after the discovery of the double helix before it was worked out. In Chapter 6 we describe this code in detail in the course of elaborating the process, known as *gene expression*, through which a cell translates the nucleotide sequence of a gene into the amino acid sequence of a protein.

The complete set of information in an organism's DNA is called its **genome**, and it carries the information for all the proteins the organism will ever synthesize. (The term genome is also used to describe the DNA that carries this information.) The amount of information contained in genomes is staggering: for example, a typical human cell contains 2 meters of DNA. Written out in the four-letter nucleotide alphabet, the nucleotide sequence of a very small human gene occupies a quarter of a page of text (Figure 4–7), while the complete sequence of nucleotides in the human genome would fill more than a thousand books the size of this one. In addition to other critical information, it carries the instructions for about 30,000 distinct proteins.

At each cell division, the cell must copy its genome to pass it to both daughter cells. The discovery of the structure of DNA also revealed the principle that makes this copying possible: because each strand of DNA contains a sequence of nucleotides that is exactly complementary to the nucleotide sequence of its partner strand, each strand can act as a **template**, or mold, for the synthesis of a new complementary strand. In other words, if we designate the two DNA strands as S and S′, strand S can serve as a template for making a new strand S′, while strand S′ can serve as a template for making a new strand S (Figure 4–8).

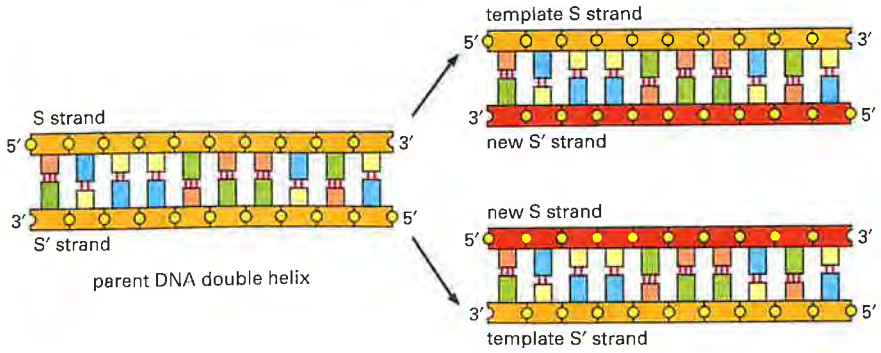


Figure 4–8 DNA as a template for its own duplication. As the nucleotide A successfully pairs only with T, and G with C, each strand of DNA can specify the sequence of nucleotides in its complementary strand. In this way, double-helical DNA can be copied precisely.

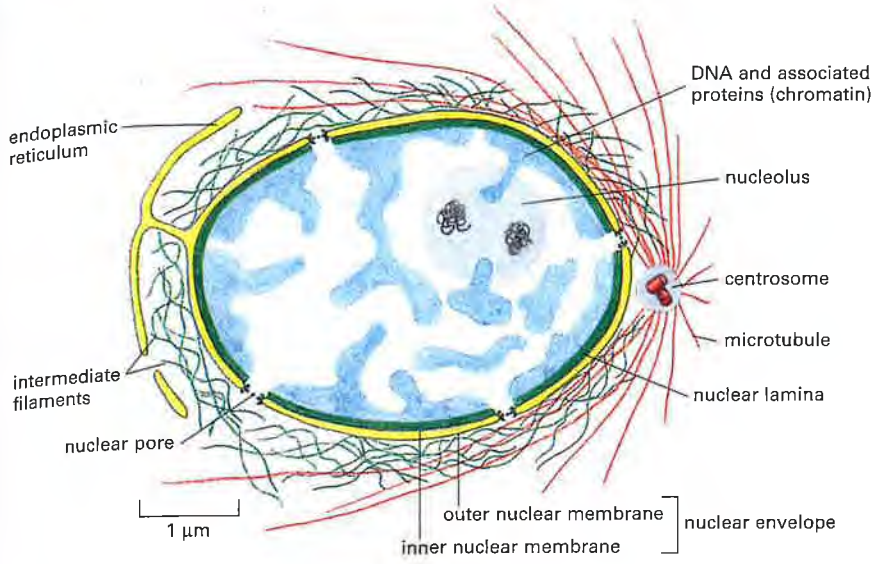


Figure 4–9 A cross-sectional view of a typical cell nucleus. The nuclear envelope consists of two membranes, the outer one being continuous with the endoplasmic reticulum membrane (see also Figure 12–9). The space inside the endoplasmic reticulum (the ER lumen) is colored *yellow*; it is continuous with the space between the two nuclear membranes. The lipid bilayers of the inner and outer nuclear membranes are connected at each nuclear pore. Two networks of intermediate filaments *(green)* provide mechanical support for the nuclear envelope; the intermediate filaments inside the nucleus form a special supporting structure called the nuclear lamina.

Thus, the genetic information in DNA can be accurately copied by the beautifully simple process in which strand S separates from strand S′, and each separated strand then serves as a template for the production of a new complementary partner strand that is identical to its former partner.

The ability of each strand of a DNA molecule to act as a template for producing a complementary strand enables a cell to copy, or *replicate*, its genes before passing them on to its descendants. In the next chapter we describe the elegant machinery the cell uses to perform this enormous task.

In Eucaryotes, DNA Is Enclosed in a Cell Nucleus

Nearly all the DNA in a eucaryotic cell is sequestered in a nucleus, which occupies about 10% of the total cell volume. This compartment is delimited by a *nuclear envelope* formed by two concentric lipid bilayer membranes that are punctured at intervals by large nuclear pores, which transport molecules between the nucleus and the cytosol. The nuclear envelope is directly connected to the extensive membranes of the endoplasmic reticulum. It is mechanically supported by two networks of intermediate filaments: one, called the *nuclear lamina*, forms a thin sheetlike meshwork inside the nucleus, just beneath the inner nuclear membrane; the other surrounds the outer nuclear membrane and is less regularly organized (Figure 4–9).

The nuclear envelope allows the many proteins that act on DNA to be concentrated where they are needed in the cell, and, as we see in subsequent chapters, it also keeps nuclear and cytosolic enzymes separate, a feature that is crucial for the proper functioning of eucaryotic cells. Compartmentalization, of which the nucleus is an example, is an important principle of biology; it serves to establish an environment in which biochemical reactions are facilitated by the high concentration of both substrates and the enzymes that act on them.

Summary

Genetic information is carried in the linear sequence of nucleotides in DNA. Each molecule of DNA is a double helix formed from two complementary strands of nucleotides held together by hydrogen bonds between G-C and A-T base pairs. Duplication of the genetic information occurs by the use of one DNA strand as a template for formation of a complementary strand. The genetic information stored in an organism's DNA contains the instructions for all the proteins the organism will ever synthesize. In eucaryotes, DNA is contained in the cell nucleus.

CHROMOSOMAL DNA AND ITS PACKAGING IN THE CHROMATIN FIBER

The most important function of DNA is to carry genes, the information that specifies all the proteins that make up an organism—including information about when, in what types of cells, and in what quantity each protein is to be made. The genomes of eucaryotes are divided up into chromosomes, and in this section we see how genes are typically arranged on each chromosome. In addition, we describe the specialized DNA sequences that allow a chromosome to be accurately duplicated and passed on from one generation to the next.

We also confront the serious challenge of DNA packaging. Each human cell contains approximately 2 meters of DNA if stretched end-to-end; yet the nucleus of a human cell, which contains the DNA, is only about 6 μm in diameter. This is geometrically equivalent to packing 40 km (24 miles) of extremely fine thread into a tennis ball! The complex task of packaging DNA is accomplished by specialized proteins that bind to and fold the DNA, generating a series of coils and loops that provide increasingly higher levels of organization, preventing the DNA from becoming an unmanageable tangle. Amazingly, although the DNA is very tightly folded, it is compacted in a way that allows it to easily become available to the many enzymes in the cell that replicate it, repair it, and use its genes to produce proteins.

Eucaryotic DNA Is Packaged into a Set of Chromosomes

In eucaryotes, the DNA in the nucleus is divided between a set of different **chromosomes**. For example, the human genome—approximately 3.2×10^9 nucleotides—is distributed over 24 different chromosomes. Each chromosome consists of a single, enormously long linear DNA molecule associated with proteins that fold and pack the fine DNA thread into a more compact structure. The complex of DNA and protein is called *chromatin* (from the Greek *chroma*, "color," because of its staining properties). In addition to the proteins involved in packaging the DNA, chromosomes are also associated with many proteins required for the processes of gene expression, DNA replication, and DNA repair.

Bacteria carry their genes on a single DNA molecule, which is usually circular (see Figure 1–30). This DNA is associated with proteins that package and condense the DNA, but they are different from the proteins that perform these functions in eucaryotes. Although often called the bacterial "chromosome," it does not have the same structure as eucaryotic chromosomes, and less is known about how the bacterial DNA is packaged. Even less is known about how DNA is compacted in archaea. Therefore, our discussion of chromosome structure will focus almost entirely on eucaryotic chromosomes.

With the exception of the germ cells, and a few highly specialized cell types that cannot multiply and lack DNA altogether (for example, red blood cells), each human cell contains two copies of each chromosome, one inherited from the mother and one from the father. The maternal and paternal chromosomes of a pair are called **homologous chromosomes (homologs)**. The only nonhomologous chromosome pairs are the sex chromosomes in males, where a *Y chromosome* is inherited from the father and an *X chromosome* from the mother. Thus, each human cell contains a total of 46 chromosomes—22 pairs common to both males and females, plus two so-called sex chromosomes (X and Y in males, two Xs in females). *DNA hybridization* (described in detail in Chapter 8) can be used to distinguish these human chromosomes by "painting" each one a different color (Figure 4–10). Chromosome painting is typically done at the stage in the cell cycle when chromosomes are especially compacted and easy to visualize (mitosis, see below).

Another more traditional way to distinguish one chromosome from another is to stain them with dyes that produce a striking and reliable pattern of bands along each mitotic chromosome (Figure 4–11). The structural bases for these banding patterns are not well understood, and we return to this issue at the end of the chapter. Nevertheless, the pattern of bands on each type of chromosome is unique, allowing each chromosome to be identified and numbered.

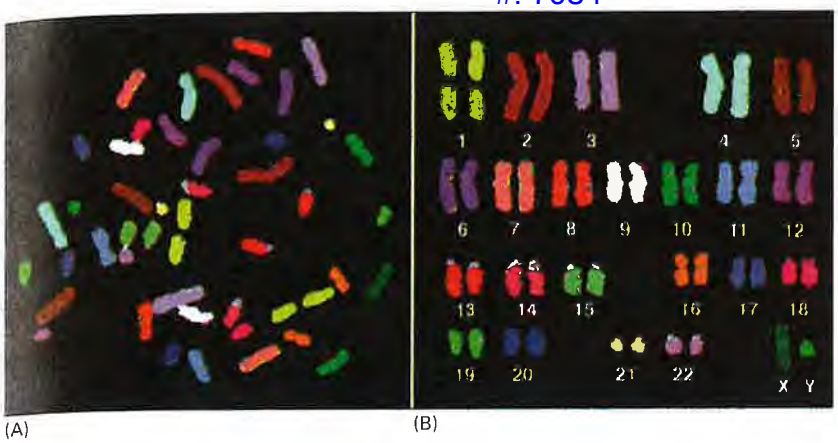


Figure 4–10 Human chromosomes. These chromosomes, from a male, were isolated from a cell undergoing nuclear division (mitosis) and are therefore highly compacted. Each chromosome has been "painted" a different color to permit its unambiguous identification under the light microscope. Chromosome painting is performed by exposing the chromosomes to a collection of human DNA molecules that have been coupled to a combination of fluorescent dyes. For example, DNA molecules derived from chromosome 1 are labeled with one specific dye combination, those from chromosome 2 with another, and so on. Because the labeled DNA can form base pairs, or hybridize, only to the chromosome from which it was derived (discussed in Chapter 8), each chromosome is differently labeled. For such experiments, the chromosomes are subjected to treatments that separate the double-helical DNA into individual strands, designed to permit base-pairing with the single-stranded labeled DNA while keeping the chromosome structure relatively intact. (A) The chromosomes visualized as they originally spilled from the lysed cell. (B) The same chromosomes artificially lined up in their numerical order. This arrangement of the full chromosome set is called a karyotype. (From E. Schröck et al., *Science* 273:494–497, 1996. © AAAS.)

The display of the 46 human chromosomes at mitosis is called the human **karyotype**. If parts of chromosomes are lost, or switched between chromosomes, these changes can be detected by changes in the banding patterns or by changes in the pattern of chromosome painting (Figure 4–12). Cytogeneticists use these alterations to detect chromosome abnormalities that are associated with inherited defects or with certain types of cancer that arise through the rearrangement of chromosomes in somatic cells.

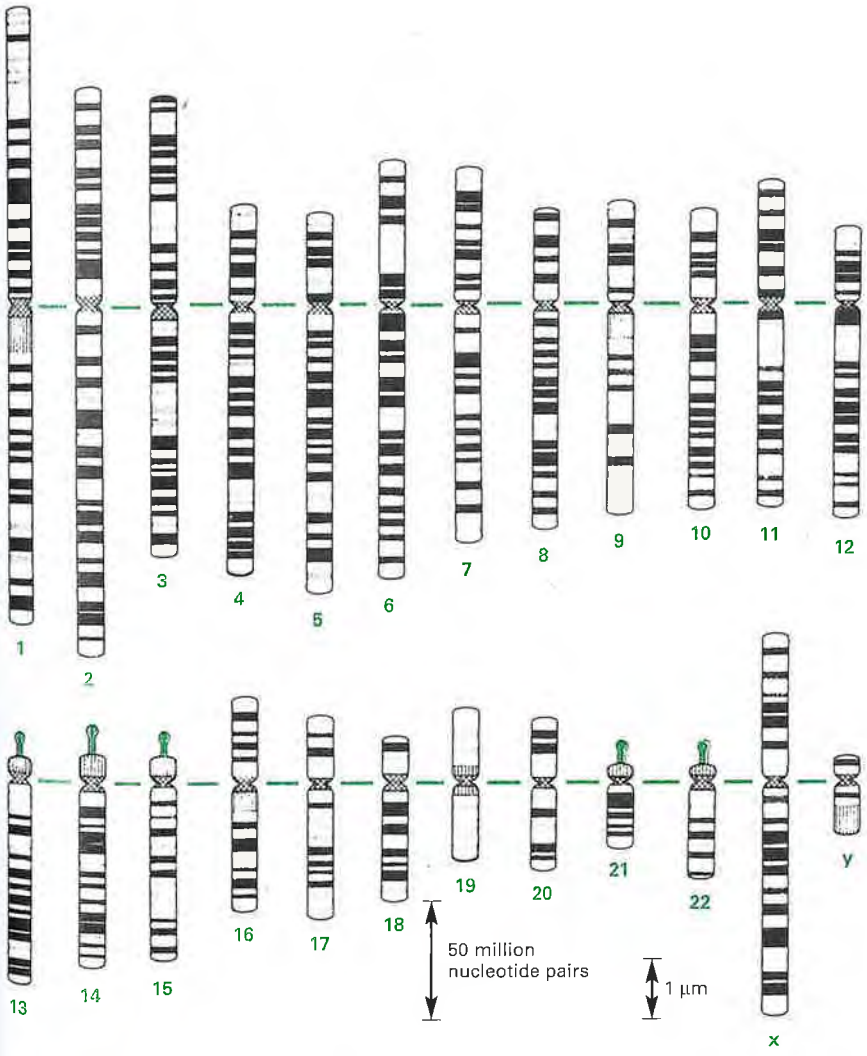


Figure 4–11 The banding patterns of human chromosomes. Chromosomes 1–22 are numbered in approximate order of size. A typical human somatic (non-germ line) cell contains two of each of these chromosomes, plus two sex chromosomes—two X chromosomes in a female, one X and one Y chromosome in a male. The chromosomes used to make these maps were stained at an early stage in mitosis, when the chromosomes are incompletely compacted. The *horizontal green line* represents the position of the centromere (see Figure 4–22), which appears as a constriction on mitotic chromosomes; the knobs on chromosomes 13, 14, 15, 21, and 22 indicate the positions of genes that code for the large ribosomal RNAs (discussed in Chapter 6). These patterns are obtained by staining chromosomes with Giemsa stain, and they can be observed under the light microscope. (Adapted from U. Franke, *Cytogenet. Cell Genet.* 31:24–32, 1981.)

Chromosomes Contain Long Strings of Genes

The most important function of chromosomes is to carry genes—the functional units of heredity. A gene is usually defined as a segment of DNA that contains the instructions for making a particular protein (or a set of closely related proteins). Although this definition holds for the majority of genes, several percent of genes produce an RNA molecule, instead of a protein, as their final product. Like proteins, these RNA molecules perform a diverse set of structural and catalytic functions in the cell, and we discuss them in detail in subsequent chapters.

As might be expected, a correlation exists between the complexity of an organism and the number of genes in its genome (see Table 1–1). For example, total gene numbers range from less than 500 for simple bacteria to about 30,000 for humans. Bacteria and some single-celled eucaryotes have especially compact genomes; the complete nucleotide sequence of their genomes reveals that the DNA molecules that make up their chromosomes are little more than strings of closely packed genes (Figure 4–13; see also Figure 1–30). However, chromosomes from many eucaryotes (including humans) contain, in addition to genes, a large excess of interspersed DNA that does not seem to carry critical information. Sometimes called junk DNA to signify that its usefulness to the cell has not been demonstrated, the particular nucleotide sequence of this DNA may not be important; but the DNA itself, by acting as spacer material, may be crucial for the long-term evolution of the species and for the proper expression of genes. These issues are taken up in detail in Chapter 7.

In general, the more complex the organism, the larger its genome, but because of differences in the amount of excess DNA, the relationship is not systematic (see Figure 1–38). For example, the human genome is 200 times larger than that of the yeast *S. cerevisiae*, but 30 times smaller than that of some plants and amphibians and 200 times smaller than a species of amoeba. Moreover, because of differences in the amount of excess DNA, the genomes of similar organisms (bony fish, for example) can vary several hundredfold in their DNA content, even though they contain roughly the same number of genes. Whatever the excess DNA may do, it seems clear that it is not a great handicap for a higher eucaryotic cell to carry a large amount of it.

The apportionment of the genome over chromosomes also differs from one eucaryotic species to the next. For example, compared with 46 for humans,

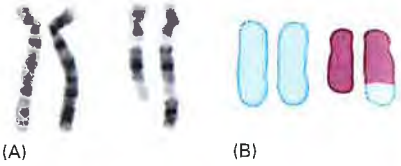


Figure 4–12 An aberrant human chromosome. (A) Two pairs of chromosomes, stained with Giemsa (see Figure 4–11), from a patient with ataxia, a disease characterized by progressive deterioration of motor skills. The patient has a normal pair of chromosome 4s *(left-hand pair)*, but one normal chromosome 12 and one aberrant chromosome 12, as seen by its greater length *(right-hand pair)*. The additional material contained on the aberrant chromosome 12 was deduced, from its pattern of bands, as a piece of chromosome 4 that had become attached to chromosome 12 through an abnormal recombination event, called a chromosomal translocation. (B) The same two chromosome pairs, "painted" *blue* for chromosome 4 DNA and *purple* for chromosome 12 DNA. The two techniques give rise to the same conclusion regarding the nature of the aberrant chromosome 12, but chromosome painting provides better resolution, and the clear identification of even short pieces of chromosomes that have become translocated. However, Giemsa staining is easier to perform. (From E. Schröck et al., *Science* 273:494–497, 1996. © AAAS.)

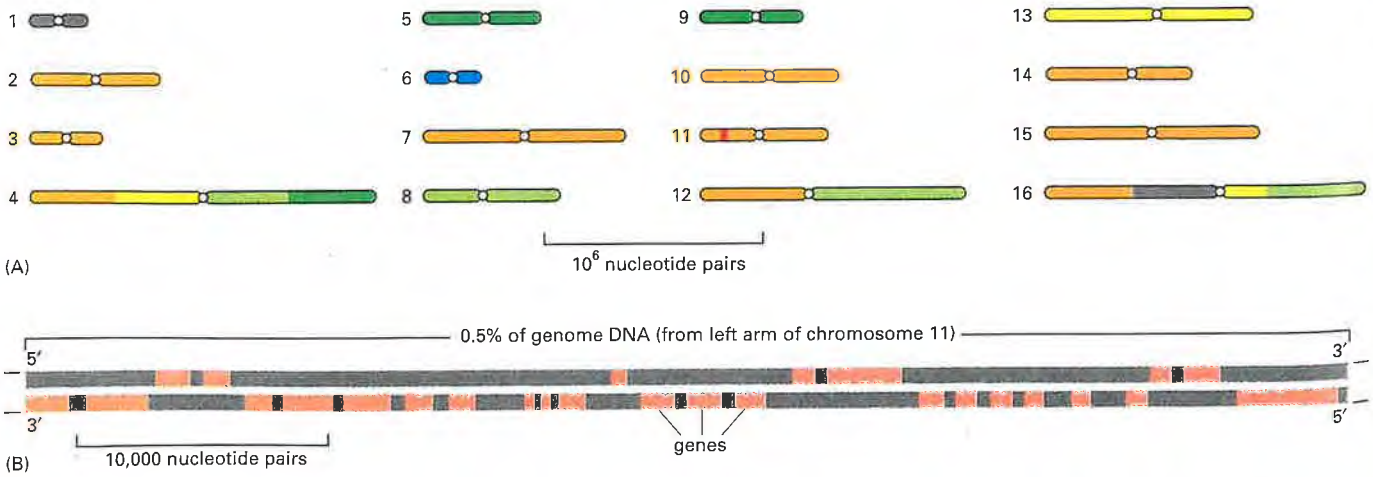


Figure 4–13 The genome of *S. cerevisiae* (budding yeast). (A) The genome is distributed over 16 chromosomes, and its complete nucleotide sequence was determined by a cooperative effort involving scientists working in many different locations, as indicated *(gray,* Canada; *orange,* European Union; *yellow,* United Kingdom; *blue,* Japan; *light green,* St Louis, Missouri; *dark green,* Stanford, California). The constriction present on each chromosome represents the position of its centromere (see Figure 4–22). (B) A small region of chromosome 11, highlighted in *red* in part A, is magnified to show the high density of genes characteristic of this species. As indicated by *orange,* some genes are transcribed from the lower strand (see Figure 1–5), while others are transcribed from the upper strand. There are about 6000 genes in the complete genome, which is 12,147,813 nucleotide pairs long.

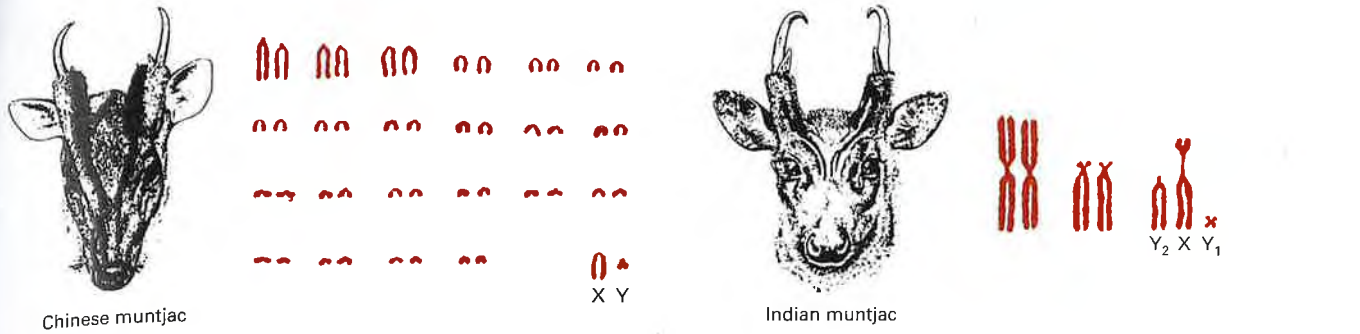


Figure 4–14 Two closely related species of deer with very different chromosome numbers. In the evolution of the Indian muntjac, initially separate chromosomes fused, without having a major effect on the animal. These two species have roughly the same number of genes. (Adapted from M.W. Strickberger, Evolution, 3rd edition, 2000, Sudbury, MA: Jones & Bartlett Publishers.)

somatic cells from a species of small deer contain only 6 chromosomes, while those from a species of carp contain over 100. Even closely related species with similar genome sizes can have very different numbers and sizes of chromosomes (Figure 4–14). Thus, there is no simple relationship between chromosome number, species complexity, and total genome size. Rather, the genomes and chromosomes of modern-day species have each been shaped by a unique history of seemingly random genetic events, acted on by selection pressures.

The Nucleotide Sequence of the Human Genome Shows How Genes Are Arranged in Humans

When the DNA sequence of human chromosome 22, one of the smallest human chromosomes (see Figure 4–11), was completed in 1999, it became possible for the first time to see exactly how genes are arranged along an entire vertebrate chromosome (Figure 4–15 and Table 4–1). With the publication of the "first draft" of the entire human genome in 2001, the genetic landscape of all human chromosomes suddenly came into sharp focus. The sheer quantity of information

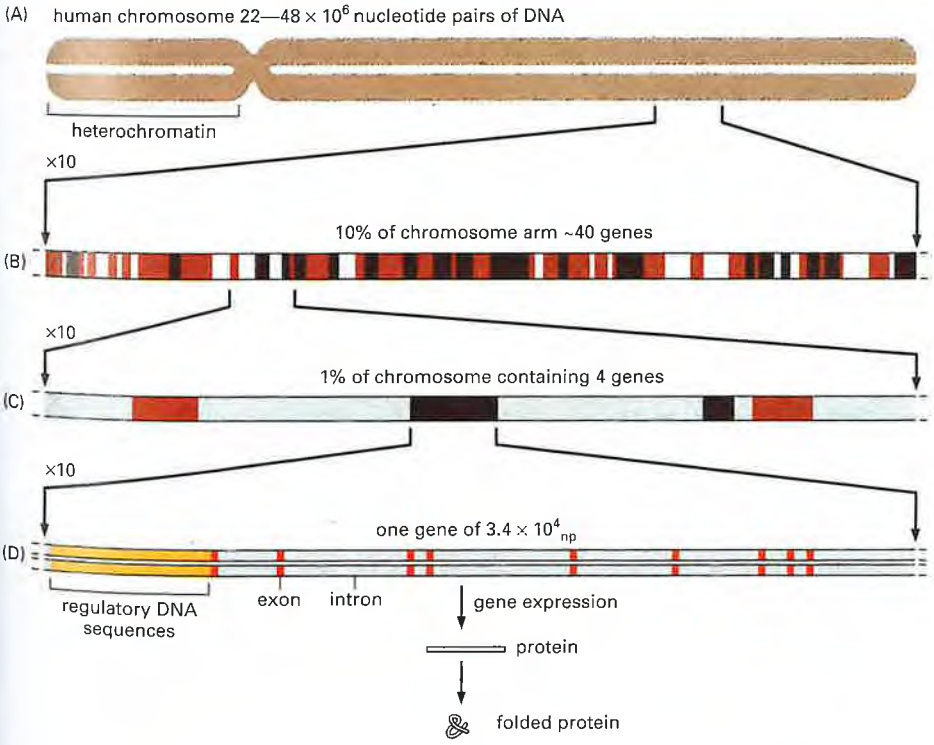


Figure 4–15 The organization of genes on a human chromosome. (A) Chromosome 22, one of the smallest human chromosomes, contains 48 × 10^6 nucleotide pairs and makes up approximately 1.5% of the entire human genome. Most of the left arm of chromosome 22 consists of short repeated sequences of DNA that are packaged in a particularly compact form of chromatin (heterochromatin), which is discussed later in this chapter. (B) A tenfold expansion of a portion of chromosome 22, with about 40 genes indicated. Those in *dark brown* are known genes and those in *light brown* are predicted genes. (C) An expanded portion of (B) shows the entire length of several genes. (D) The intron–exon arrangement of a typical gene is shown after a further tenfold expansion. Each exon *(red)* codes for a portion of the protein, while the DNA sequence of the introns *(gray)* is relatively unimportant. The entire human genome (3.2 × 10^9 nucleotide pairs) is distributed over 22 autosomes and 2 sex chromosomes (see Figures 4–10 and 4–11). The term *human genome sequence* refers to the complete nucleotide sequence of DNA in these 24 chromosomes. Being diploid, a human somatic cell therefore contains roughly twice this amount of DNA. Humans differ from one another by an average of one nucleotide in every thousand, and a wide variety of humans contributed DNA for the genome sequencing project. The published human genome sequence is therefore a composite of many individual sequences. (Adapted from International Human Genome Sequencing Consortium, *Nature* 409:860–921, 2001.)

provided by the Human Genome Project is unprecedented in biology (Figure 4–16 and Table 4–1); the human genome is 25 times larger than any other genome sequenced so far, and is 8 times as large as the sum of all previously sequenced genomes. At its peak, the Human Genome Project generated raw nucleotide sequences at a rate of 1000 nucleotides per second around the clock. It will be many decades before this information is fully analyzed, but it will continue to stimulate many new experiments and has already affected the content of all the chapters in this book.

Although there are many aspects to analyzing the human genome, here we simply make a few generalizations regarding the arrangement of genes in human chromosomes. The first striking feature of the human genome is how little of it (only a few percent) codes for proteins or structural and catalytic RNAs (Figure 4–17). Much of the remaining chromosomal DNA is made up of short, mobile pieces of DNA that have gradually inserted themselves in the chromosome over evolutionary time. We discuss these *transposable elements* in detail in later chapters.

A second notable feature of the human genome is the large average gene size of 27,000 nucleotide pairs. As discussed above, a typical gene carries in its linear sequence of nucleotides the information for the linear sequence of the amino acids of a protein. Only about 1300 nucleotide pairs are required to encode a protein of average size (about 430 amino acids in humans). Most of the remaining DNA in a gene consists of long stretches of noncoding DNA that interrupt the relatively short segments of DNA that code for protein. The coding sequences are called *exons*; the intervening (noncoding) sequences are called *introns* (see Figure 4–15 and Table 4–1).

The majority of human genes thus consist of a long string of alternating exons and introns, with most of the gene consisting of introns. In contrast, the majority of genes from organisms with compact genomes lack introns. This accounts for the much smaller size of their genes (about one-twentieth that of human genes), as well as for the much higher fraction of coding DNA in their chromosomes. In addition to introns and exons, each gene is associated with *regulatory DNA sequences*, which are responsible for ensuring that the gene is

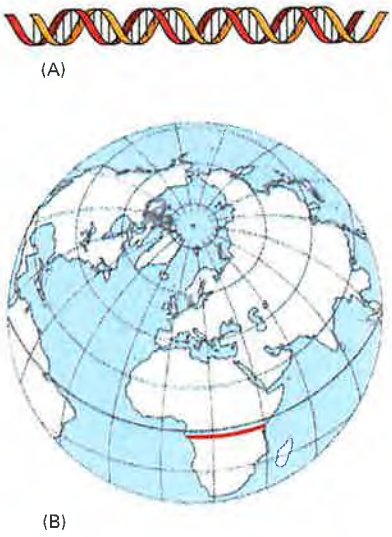


Figure 4–16 Scale of the human genome. If each nucleotide pair is drawn as 1 mm as in (A), then the human genome would extend 3200 km (approximately 2000 miles), far enough to stretch across the center of Africa, the site of our human origins (*red line* in B). At this scale, there would be, on average, a protein-coding gene every 300 m. An average gene would extend for 30 m, but the coding sequences in this gene would add up to only just over a meter.

TABLE 4–1 Vital Statistics of Human Chromosome 22 and the Entire Human Genome

	CHROMOSOME 22	HUMAN GENOME
DNA length	48×10^6 nucleotide pairs*	3.2×10^9
Number of genes	approximately 700	approximately 30,000
Smallest protein-coding gene	1000 nucleotide pairs	not analyzed
Largest gene	583,000 nucleotide pairs	2.4×10^6 nucleotide pairs
Mean gene size	19,000 nucleotide pairs	27,000 nucleotide pairs
Smallest number of exons per gene	1	1
Largest number of exons per gene	54	178
Mean number of exons per gene	5.4	8.8
Smallest exon size	8 nucleotide pairs	not analyzed
Largest exon size	7600 nucleotide pairs	17,106 nucleotide pairs
Mean exon size	266 nucleotide pairs	145 nucleotide pairs
Number of pseudogenes**	more than 134	not analyzed
Percentage of DNA sequence in exons (protein coding sequences)	3%	1.5%
Percentage of DNA in high-copy repetitive elements	42%	approximately 50%
Percentage of total human genome	1.5%	100%

* The nucleotide sequence of 33.8×10^6 nucleotides is known; the rest of the chromosome consists primarily of very short repeated sequences that do not code for proteins or RNA.

** A pseudogene is a nucleotide sequence of DNA closely resembling that of a functional gene, but containing numerous deletion mutations that prevent its proper expression. Most pseudogenes arise from the duplication of a functional gene followed by the accumulation of damaging mutations in one copy.

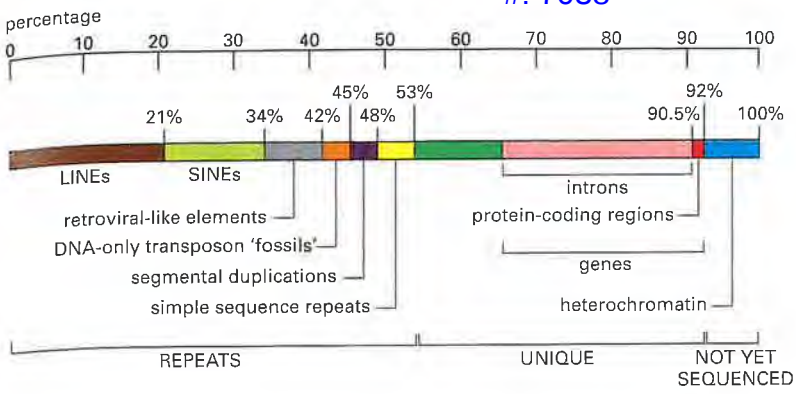


Figure 4–17 Representation of the nucleotide sequence content of the human genome. LINES, SINES, retroviral-like elements, and DNA-only transposons are all mobile genetic elements that have multiplied in our genome by replicating themselves and inserting the new copies in different positions. Mobile genetic elements are discussed in Chapter 5. Simple sequence repeats are short nucleotide sequences (less than 14 nucleotide pairs) that are repeated again and again for long stretches. Segmental duplications are large blocks of the genome (1000–200,000 nucleotide pairs) that are present at two or more locations in the genome. Over half of the unique sequence consists of genes and the remainder is probably regulatory DNA. Most of the DNA present in heterochromatin, a specialized type of chromatin (discussed later in this chapter) that contains relatively few genes, has not yet been sequenced. (Adapted from Unveiling the Human Genome, Supplement to the Wellcome Trust Newsletter. London: Wellcome Trust, February 2001.)

expressed at the proper level and time, and the proper type of cell. In humans, the regulatory sequences for a typical gene are spread out over tens of thousands of nucleotide pairs. As would be expected, these regulatory sequences are more compressed in organisms with compact genomes. We discuss in Chapter 7 how regulatory DNA sequences work.

Finally, the nucleotide sequence of the human genome has revealed that the critical information seems to be in an alarming state of disarray. As one commentator described our genome, "In some ways it may resemble your garage/bedroom/refrigerator/life: highly individualistic, but unkempt; little evidence of organization; much accumulated clutter (referred to by the uninitiated as 'junk'); virtually nothing ever discarded; and the few patently valuable items indiscriminately, apparently carelessly, scattered throughout."

Comparisons Between the DNAs of Related Organisms Distinguish Conserved and Nonconserved Regions of DNA Sequence

A major obstacle in interpreting the nucleotide sequences of human chromosomes is the fact that much of the sequence is probably unimportant. Moreover, the coding regions of the genome (the exons) are typically found in short segments (average size about 145 nucleotide pairs) floating in a sea of DNA whose exact nucleotide sequence is of little consequence. This arrangement makes it very difficult to identify all the exons in a stretch of DNA sequence; even harder is the determination of where a gene begins and ends and how many exons it spans. Accurate gene identification requires approaches that extract information from the inherently low signal-to-noise ratio of the human genome, and we describe some of them in Chapter 8. Here we discuss the most general approach, one that has the potential to identify not only coding sequences but also additional DNA sequences that are important. It is based on the observation that sequences that have a function are conserved during evolution, whereas those without a function are free to mutate randomly. The strategy is therefore to compare the human sequence with that of the corresponding regions of a related genome, such as that of the mouse. Humans and mice are thought to have diverged from a common mammalian ancestor about 100×10^6 years ago, which is long enough for the majority of nucleotides in their genomes to have been changed by random mutational events. Consequently, the only regions that will have remained closely similar in the two genomes are those in which mutations would have impaired function and put the animals carrying them at a disadvantage, resulting in their elimination from the population by natural selection. Such closely similar regions are known as *conserved regions*. In general, conserved regions represent functionally important exons and regulatory sequences. In contrast, *nonconserved regions* represent DNA whose sequence is generally not critical for function. By revealing in this way the results of a very long natural "experiment," comparative DNA sequencing studies highlight the most interesting regions in genomes.

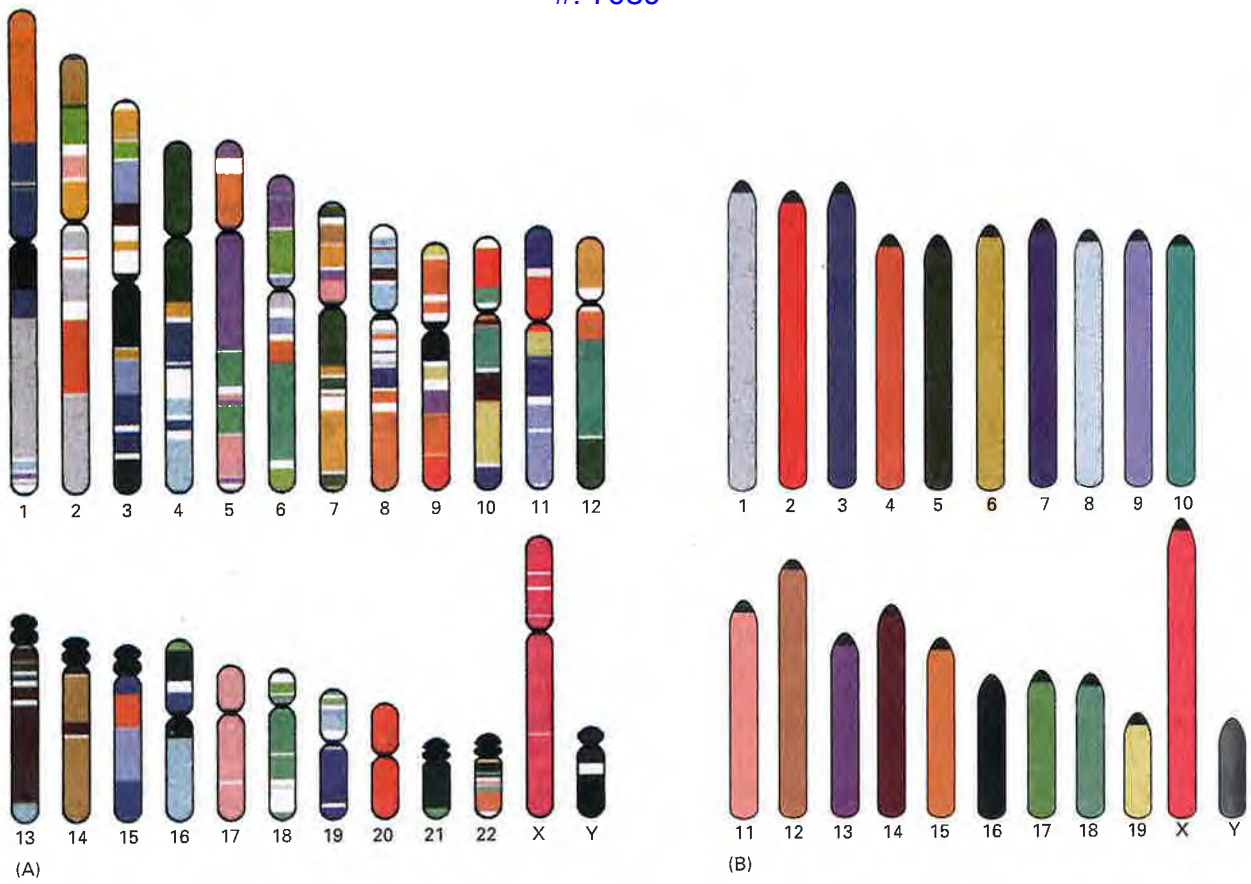


Figure 4–18 Conserved synteny between the human and mouse genomes. Regions from different mouse chromosomes (indicated by the colors of each mouse in B) show conserved synteny (gene order) with the indicated regions of the human genome (A). For example the genes present in the upper portion of human chromosome 1 *(orange)* are present in the same order in a portion of mouse chromosome 4. Regions of human chromosomes that are composed primarily of short, repeated sequences are shown in *black*. Mouse centromeres (indicated in *black* in B) are located at the ends of chromosomes; no known genes lie beyond the centromere on any mouse chromosome. For the most part, human centromeres, indicated by constrictions, occupy more internal positions on chromosomes (see Figure 4–11). (Adapted from International Human Genome Sequencing Consortium, *Nature* 409:860–921, 2001.)

Comparative studies of this kind have revealed not only that mice and humans share most of the same genes, but also that large blocks of the mouse and human genomes contain these genes in the same order, a feature called *conserved synteny* (Figure 4–18). Conserved synteny can also be revealed by chromosome painting, and this technique has been used to reconstruct the evolutionary history of our own chromosomes by comparing them with those from other mammals (Figure 4–19).

Chromosomes Exist in Different States Throughout the Life of a Cell

We have seen how genes are arranged in chromosomes, but to form a functional chromosome, a DNA molecule must be able to do more than simply carry genes: it must be able to replicate, and the replicated copies must be separated and reliably partitioned into daughter cells at each cell division. This process occurs through an ordered series of stages, collectively known as the **cell cycle**. The cell cycle is briefly summarized in Figure 4–20, and discussed in detail in Chapter 17. Only two of the stages of the cycle concern us in this chapter. During *interphase* chromosomes are replicated, and during *mitosis* they become highly condensed and then are separated and distributed to the two daughter nuclei. The highly condensed chromosomes in a dividing cell are known as *mitotic chromosomes*. This is the form in which chromosomes are most easily visualized; in fact, all the images of chromosomes shown so far in the chapter are of chromosomes in mitosis. This condensed state is important in allowing the duplicated chromosomes to be separated by the mitotic spindle during cell division, as discussed in Chapter 18.

During the portions of the cell cycle when the cell is not dividing, the chromosomes are extended and much of their chromatin exists as long, thin tangled

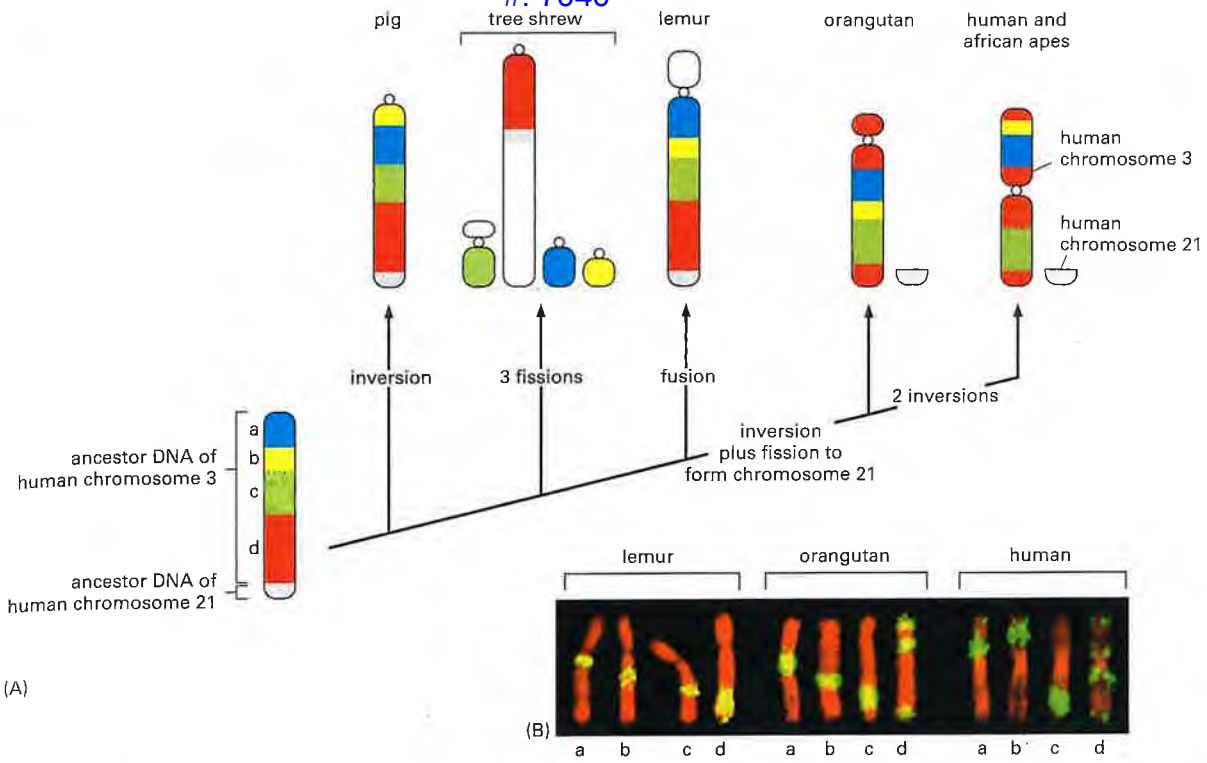


Figure 4–19 A proposed evolutionary history of human chromosome 3 and its relatives in other mammals. (A) At the *lower left* is the order of chromosome 3 segments hypothesized to be present on a chromosome of a mammalian ancestor. Along the *top* are the patterns of chromosome sequences found in the chromosomes of modern mammals. The minimum changes necessary to account for the appearance of the modern chromosomes from the hypothetical ancestor are marked along each branch. In mammals, these types of changes in chromosome organization are thought to occur once every 5–10 × 10^6 years. The *small circles* depicted in the modern chromosomes represent the positions of centromeres. (B) Some of the chromosome painting experiments that led to the diagram in (A). Each image shows the chromosome most closely related to human chromosome 3, painted *green* by hybridization with different segments of DNA, lettered a, b, c, and d along the *bottom* of the figure. These letters correspond to the colored segments of the diagram in (A). (From S. Müller et al., *Proc. Natl. Acad. Sci. USA* 97:206–211, 2000. © National Academy of Sciences.)

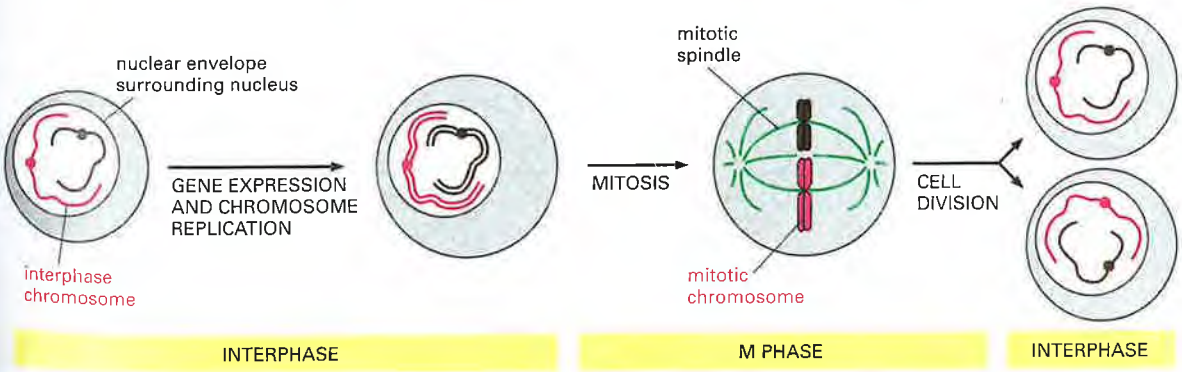


Figure 4–20 A simplified view of the eucaryotic cell cycle. During interphase, the cell is actively expressing its genes and is therefore synthesizing proteins. Also, during interphase and before cell division, the DNA is replicated and the chromosomes are duplicated. Once DNA replication is complete, the cell can enter *M phase*, when mitosis occurs and the nucleus is divided into two daughter nuclei. During this stage, the chromosomes condense, the nuclear envelope breaks down, and the mitotic spindle forms from microtubules and other proteins. The condensed mitotic chromosomes are captured by the mitotic spindle, and one complete set of chromosomes is then pulled to each end of the cell. A nuclear envelope re-forms around each chromosome set, and in the final step of M phase, the cell divides to produce two daughter cells. Most of the time in the cell cycle is spent in interphase; M phase is brief in comparison, occupying only about an hour in many mammalian cells.

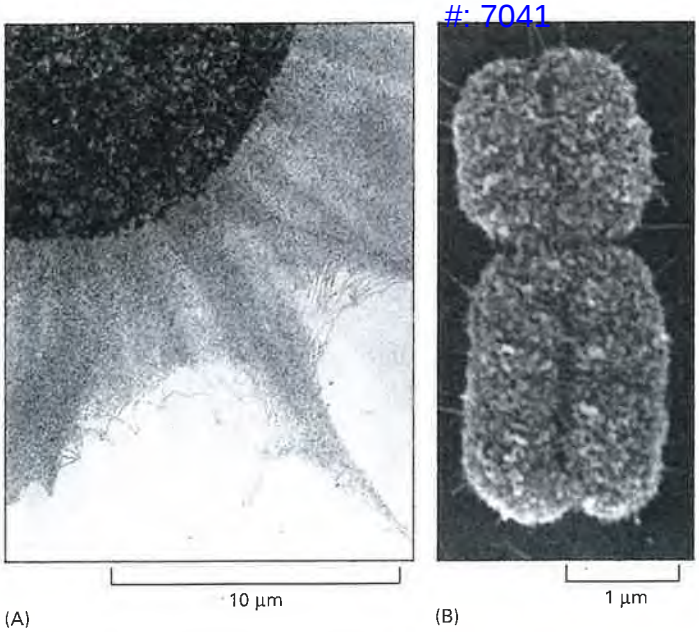


Figure 4–21 A comparison of extended interphase chromatin with the chromatin in a mitotic chromosome. (A) An electron micrograph showing an enormous tangle of chromatin spilling out of a lysed interphase nucleus. (B) A scanning electron micrograph of a mitotic chromosome: a condensed duplicated chromosome in which the two new chromosomes are still linked together (see Figure 4–22). The constricted region indicates the position of the centromere. Note the difference in scales. (A, courtesy of Victoria Foe; B, courtesy of Terry D. Allen.)

threads in the nucleus so that individual chromosomes cannot be easily distinguished (Figure 4–21). We refer to chromosomes in this extended state as *interphase chromosomes.*

Each DNA Molecule That Forms a Linear Chromosome Must Contain a Centromere, Two Telomeres, and Replication Origins

A chromosome operates as a distinct structural unit: for a copy to be passed on to each daughter cell at division, each chromosome must be able to replicate, and the newly replicated copies must subsequently be separated and partitioned correctly into the two daughter cells. These basic functions are controlled by three types of specialized nucleotide sequence in the DNA, each of which binds specific proteins that guide the machinery that replicates and segregates chromosomes (Figure 4–22).

Experiments in yeasts, whose chromosomes are relatively small and easy to manipulate, have identified the minimal DNA sequence elements responsible for each of these functions. One type of nucleotide sequence acts as a DNA **replication origin**, the location at which duplication of the DNA begins. Eucaryotic chromosomes contain many origins of replication to ensure that the entire chromosome can be replicated rapidly, as discussed in detail in Chapter 5.

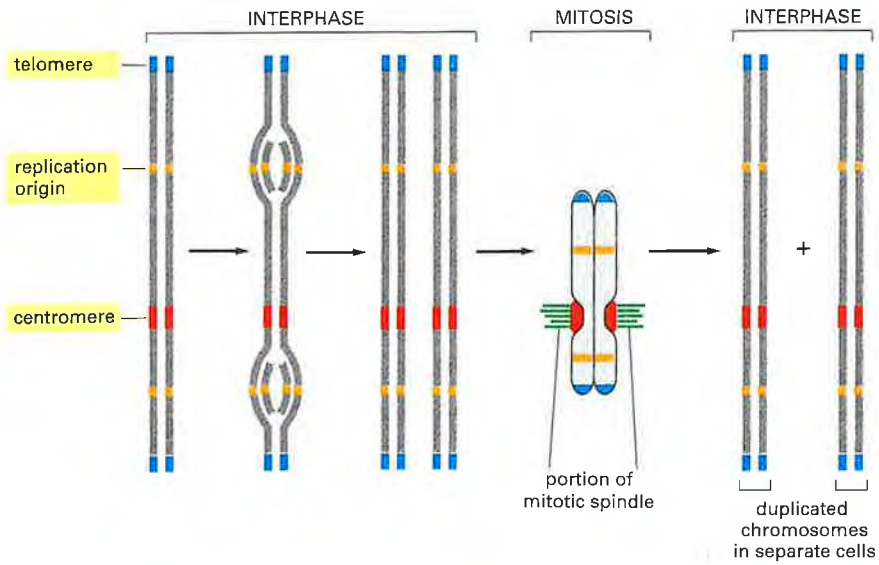


Figure 4–22 The three DNA sequences required to produce a eucaryotic chromosome that can be replicated and then segregated at mitosis. Each chromosome has multiple origins of replication, one centromere, and two telomeres. Shown here is the sequence of events a typical chromosome follows during the cell cycle. The DNA replicates in interphase beginning at the origins of replication and proceeding bidirectionally from the origins across the chromosome. In M phase, the centromere attaches the duplicated chromosomes to the mitotic spindle so that one copy is distributed to each daughter cell during mitosis. The centromere also helps to hold the duplicated chromosomes together until they are ready to be moved apart. The telomeres form special caps at each chromosome end.

After replication, the two daughter chromosomes remain attached to one another and, as the cell cycle proceeds, are condensed further to produce mitotic chromosomes. The presence of a second specialized DNA sequence, called a **centromere**, allows one copy of each duplicated and condensed chromosome to be pulled into each daughter cell when a cell divides. A protein complex called a *kinetochore* forms at the centromere and attaches the duplicated chromosomes to the mitotic spindle, allowing them to be pulled apart (discussed in Chapter 18).

The third specialized DNA sequence forms **telomeres**, the ends of a chromosome. Telomeres contain repeated nucleotide sequences that enable the ends of chromosomes to be efficiently replicated. Telomeres also perform another function: the repeated telomere DNA sequences, together with the regions adjoining them, form structures that protect the end of the chromosome from being recognized by the cell as a broken DNA molecule in need of repair. We discuss this type of repair and the other features of telomeres in Chapter 5.

In yeast cells, the three types of sequences required to propagate a chromosome are relatively short (typically less than 1000 base pairs each) and therefore use only a tiny fraction of the information-carrying capacity of a chromosome. Although telomere sequences are fairly simple and short in all eucaryotes, the DNA sequences that specify centromeres and replication origins in more complex organisms are much longer than their yeast counterparts. For example, experiments suggest that human centromeres may contain up to 100,000 nucleotide pairs. It has been proposed that human centromeres may not even require a stretch of DNA with a defined nucleotide sequence; instead, they may simply create a large, regularly repeating protein–nucleic acid structure. We return to this issue at the end of the chapter when we discuss in more general terms the proteins that, along with DNA, make up chromosomes.

DNA Molecules Are Highly Condensed in Chromosomes

All eucaryotic organisms have elaborate ways of packaging DNA into chromosomes. Recall from earlier in this chapter that human chromosome 22 contains about 48 million nucleotide pairs. Stretched out end to end, its DNA would extend about 1.5 cm. Yet, when it exists as a mitotic chromosome, chromosome 22 measures only about 2 μm in length (see Figures 4–10 and 4–11), giving an end-to-end compaction ratio of nearly 10,000-fold. This remarkable feat of compression is performed by proteins that successively coil and fold the DNA into higher and higher levels of organization. Although less condensed than mitotic chromosomes, the DNA of interphase chromosomes is still tightly packed, with an overall compaction ratio of approximately 1000-fold. In the next sections we discuss the specialized proteins that make the compression possible.

In reading these sections it is important to keep in mind that chromosome structure is dynamic. Not only do chromosomes globally condense in accord with the cell cycle, but different regions of the interphase chromosomes condense and decondense as the cells gain access to specific DNA sequences for gene expression, DNA repair, and replication. The packaging of chromosomes must therefore be accomplished in a way that allows rapid localized, on-demand access to the DNA.

Nucleosomes Are the Basic Unit of Eucaryotic Chromosome Structure

The proteins that bind to the DNA to form eucaryotic chromosomes are traditionally divided into two general classes: the **histones** and the *nonhistone chromosomal proteins*. The complex of both classes of protein with the nuclear DNA of eucaryotic cells is known as **chromatin**. Histones are present in such enormous quantities in the cell (about 60 million molecules of each type per human cell) that their total mass in chromatin is about equal to that of the DNA.

Histones are responsible for the first and most basic level of chromosome organization, the **nucleosome**, which was discovered in 1974. When interphase

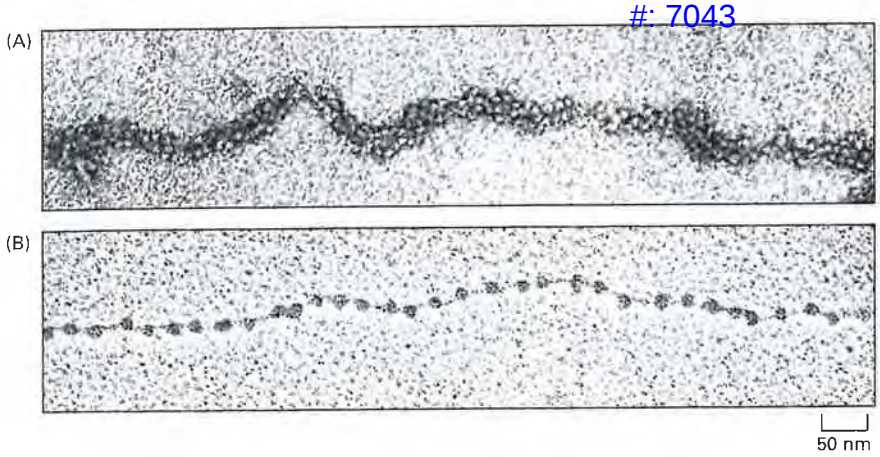


Figure 4–23 Nucleosomes as seen in the electron microscope.
(A) Chromatin isolated directly from an interphase nucleus appears in the electron microscope as a thread 30 nm thick. (B) This electron micrograph shows a length of chromatin that has been experimentally unpacked, or decondensed, after isolation to show the nucleosomes. (A, courtesy of Barbara Hamkalo; B, courtesy of Victoria Foe.)

nuclei are broken open very gently and their contents examined under the electron microscope, most of the chromatin is in the form of a fiber with a diameter of about 30 nm (Figure 4–23A). If this chromatin is subjected to treatments that cause it to unfold partially, it can be seen under the electron microscope as a series of "beads on a string" (Figure 4–23B). The string is DNA, and each bead is a "nucleosome core particle" that consists of DNA wound around a protein core formed from histones. The beads on a string represent the first level of chromosomal DNA packing.

The structural organization of nucleosomes was determined after first isolating them from unfolded chromatin by digestion with particular enzymes (called nucleases) that break down DNA by cutting between the nucleosomes. After digestion for a short period, the exposed DNA between the nucleosome core particles, the *linker DNA*, is degraded. Each individual nucleosome core particle consists of a complex of eight histone proteins—two molecules each of histones H2A, H2B, H3, and H4—and double-stranded DNA that is 146 nucleotide pairs long. The *histone octamer* forms a protein core around which the double-stranded DNA is wound (Figure 4–24).

Each nucleosome core particle is separated from the next by a region of linker DNA, which can vary in length from a few nucleotide pairs up to about 80. (The term *nucleosome* technically refers to a nucleosome core particle plus one of its adjacent DNA linkers, but it is often used synonymously with nucleosome core particle.) On average, therefore, nucleosomes repeat at intervals of about 200 nucleotide pairs. For example, a diploid human cell with 6.4×10^9 nucleotide pairs contains approximately 30 million nucleosomes. The formation of nucleosomes converts a DNA molecule into a chromatin thread about one-third of its initial length, and this provides the first level of DNA packing.

The Structure of the Nucleosome Core Particle Reveals How DNA Is Packaged

The high-resolution structure of a nucleosome core particle, solved in 1997, revealed a disc-shaped histone core around which the DNA was tightly wrapped 1.65 turns in a left-handed coil (Figure 4–25). All four of the histones that make up the core of the nucleosome are relatively small proteins (102–135 amino

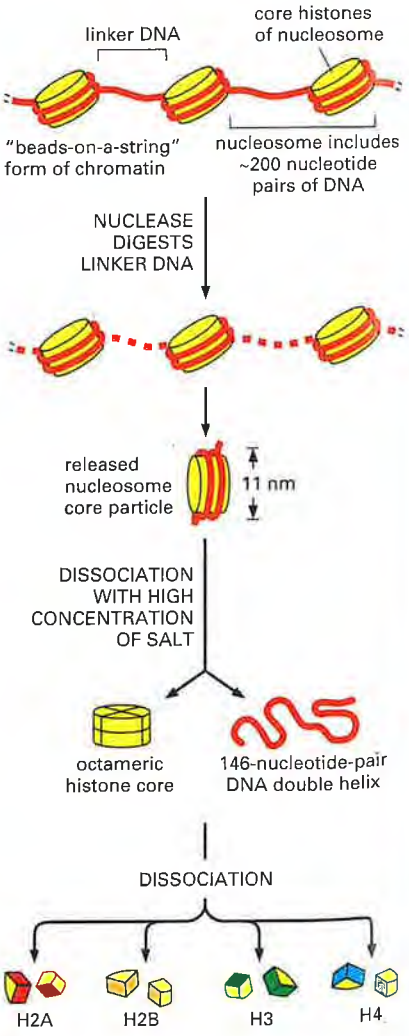


Figure 4–24 Structural organization of the nucleosome.
A nucleosome contains a protein core made of eight histone molecules. As indicated, the nucleosome core particle is released from chromatin by digestion of the linker DNA with a nuclease, an enzyme that breaks down DNA. (The nuclease can degrade the exposed linker DNA but cannot attack the DNA wound tightly around the nucleosome core.) After dissociation of the isolated nucleosome into its protein core and DNA, the length of the DNA that was wound around the core can be determined. This length of 146 nucleotide pairs is sufficient to wrap 1.65 times around the histone core.

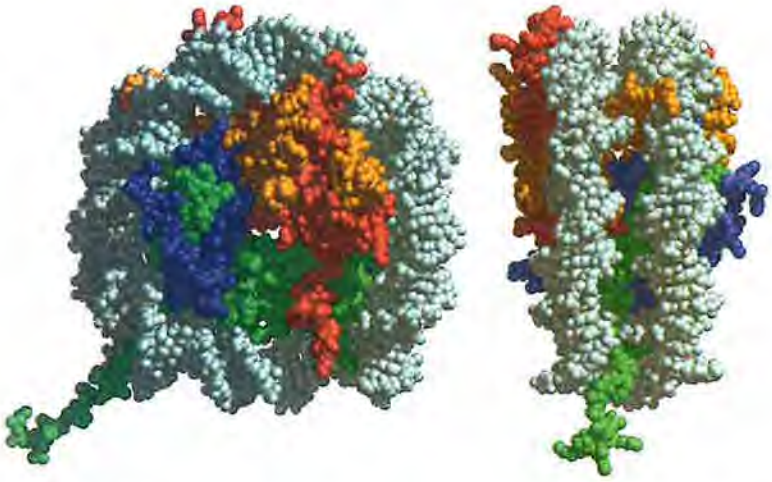


Figure 4–25 The structure of a nucleosome core particle, as determined by x-ray diffraction analyses of crystals. Each histone is colored according to the scheme of Figure 4–24, with the DNA double helix in *light gray*. (Reprinted by permission from K. Luger et al., *Nature* 389:251–260, 1997. © Macmillan Magazines Ltd.)

acids), and they share a structural motif, known as the *histone fold*, formed from three α helices connected by two loops (Figure 4–26). In assembling a nucleosome, the histone folds first bind to each other to form H3–H4 and H2A–H2B dimers, and the H3–H4 dimers combine to form tetramers. An H3–H4 tetramer then further combines with two H2A–H2B dimers to form the compact octamer core, around which the DNA is wound (Figure 4–27).

The interface between DNA and histone is extensive: 142 hydrogen bonds are formed between DNA and the histone core in each nucleosome. Nearly half of these bonds form between the amino acid backbone of the histones and the phosphodiester backbone of the DNA. Numerous hydrophobic interactions and salt linkages also hold DNA and protein together in the nucleosome. For example, all the core histones are rich in lysine and arginine (two amino acids with basic side chains), and their positive charges can effectively neutralize the negatively charged DNA backbone. These numerous interactions explain in part why DNA of virtually any sequence can be bound on a histone octamer core. The path of the DNA around the histone core is not smooth; rather, several kinks are seen in the DNA, as expected from the nonuniform surface of the core.

In addition to its histone fold, each of the core histones has a long N-terminal amino acid "tail", which extends out from the DNA-histone core (see Figure 4–27). These histone tails are subject to several different types of covalent modifications, which control many aspects of chromatin structure. We discuss these issues later in the chapter.

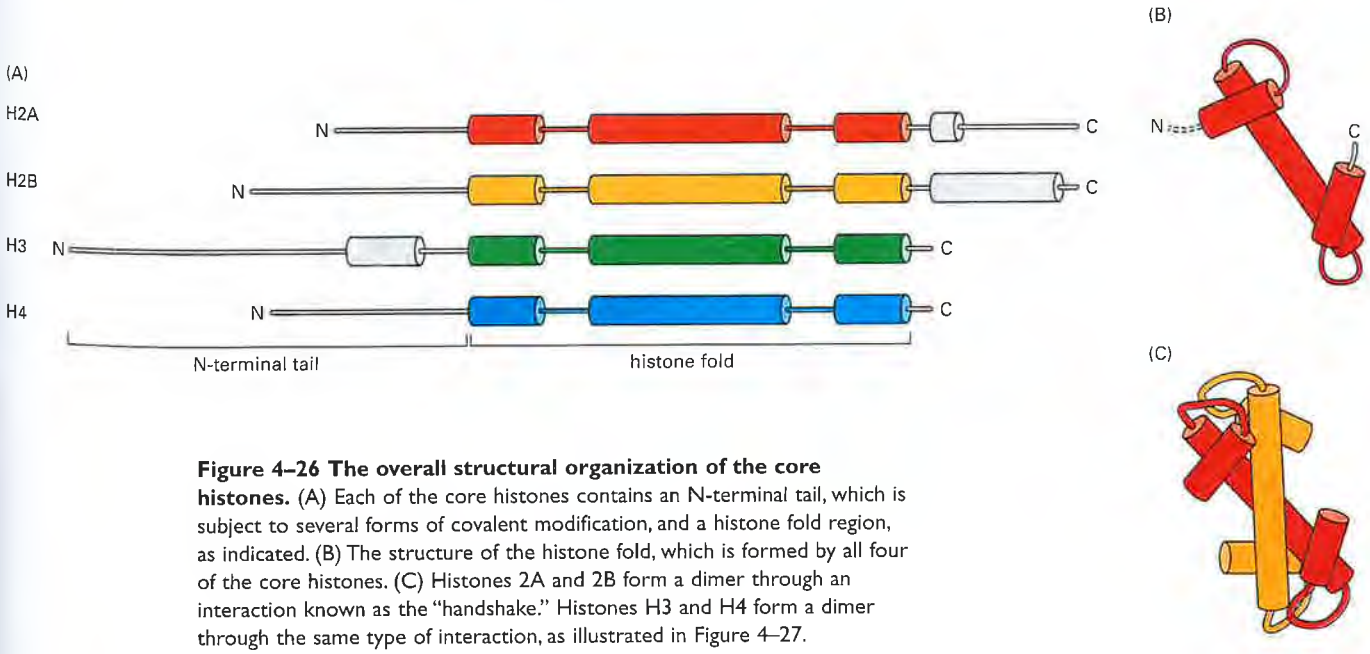


Figure 4–26 The overall structural organization of the core histones. (A) Each of the core histones contains an N-terminal tail, which is subject to several forms of covalent modification, and a histone fold region, as indicated. (B) The structure of the histone fold, which is formed by all four of the core histones. (C) Histones 2A and 2B form a dimer through an interaction known as the "handshake." Histones H3 and H4 form a dimer through the same type of interaction, as illustrated in Figure 4–27.

As might be expected from their fundamental role in DNA packaging, the histones are among the most highly conserved eucaryotic proteins. For example, the amino acid sequence of histone H4 from a pea and a cow differ at only at 2 of the 102 positions. This strong evolutionary conservation suggests that the functions of histones involve nearly all of their amino acids, so that a change in any position is deleterious to the cell. This suggestion has been tested directly in yeast cells, in which it is possible to mutate a given histone gene *in vitro* and introduce it into the yeast genome in place of the normal gene. As might be expected, most changes in histone sequences are lethal; the few that are not lethal cause changes in the normal pattern of gene expression, as well as other abnormalities.

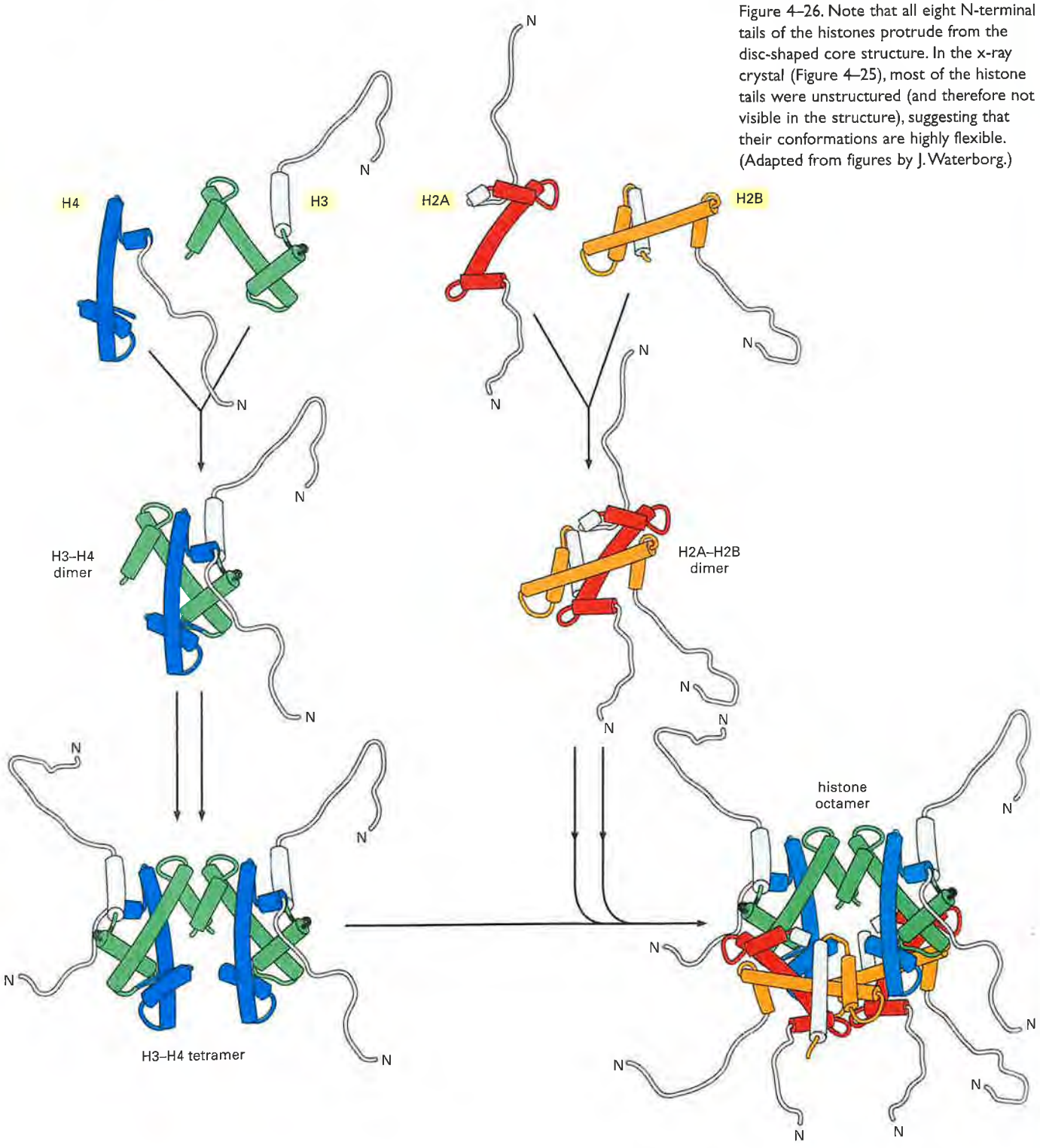


Figure 4–27 The assembly of a histone octamer. The histone H3–H4 dimer and the H2A–H2B dimer are formed from the handshake interaction. An H3–H4 tetramer forms the scaffold of the octamer onto which two H2A–H2B dimers are added, to complete the assembly. The histones are colored as in Figure 4–26. Note that all eight N-terminal tails of the histones protrude from the disc-shaped core structure. In the x-ray crystal (Figure 4–25), most of the histone tails were unstructured (and therefore not visible in the structure), suggesting that their conformations are highly flexible. (Adapted from figures by J. Waterborg.)

Despite the high conservation of the core histones, many eucaryotic organisms also produce specialized variant core histones that differ in amino acid sequence from the main ones. For example, the sea urchin has five histone H2A variants, each of which is expressed at a different time during development. It is thought that nucleosomes that have incorporated these variant histones differ in stability from regular nucleosomes, and they may be particularly well suited for the high rates of DNA transcription and DNA replication that occur during these early stages of development.

The Positioning of Nucleosomes on DNA Is Determined by Both DNA Flexibility and Other DNA-bound Proteins

Although nearly every DNA sequence can, in principle, be folded into a nucleosome, the spacing of nucleosomes in the cell can be irregular. Two main influences determine where nucleosomes form in the DNA. One is the difficulty of bending the DNA double helix into two tight turns around the outside of the histone octamer, a process that requires substantial compression of the minor groove of the DNA helix. Because A-T-rich sequences in the minor groove are easier to compress than G-C-rich sequences, each histone octamer tends to position itself on the DNA so as to maximize A-T-rich minor grooves on the inside of the DNA coil (Figure 4–28). Thus, a segment of DNA that contains short A-T-rich sequences spaced by an integral number of DNA turns is easier to bend around the nucleosome than a segment of DNA lacking this feature. In addition, because the DNA in a nucleosome is kinked in several places, the ability of a given nucleotide sequence to accommodate this deformation can also influence the position of DNA on the nucleosome.

These features of DNA probably explain some striking, but unusual, cases of very precise positioning of nucleosomes along a stretch of DNA. For most of the DNA sequences found in chromosomes, however, there is no strongly preferred nucleosome-binding site; a nucleosome can occupy any one of a number of positions relative to the DNA sequence.

The second, and probably most important, influence on nucleosome positioning is the presence of other tightly bound proteins on the DNA. Some bound proteins favor the formation of a nucleosome adjacent to them. Others create obstacles that force the nucleosomes to assemble at positions between them. Finally, some proteins can bind tightly to DNA even when their DNA-binding site is part of a nucleosome. The exact positions of nucleosomes along a stretch of DNA therefore depend on factors that include the DNA sequence and the presence and nature of other proteins bound to the DNA. Moreover, as we see below, the arrangement of nucleosomes on DNA is highly dynamic, changing rapidly according to the needs of the cell.

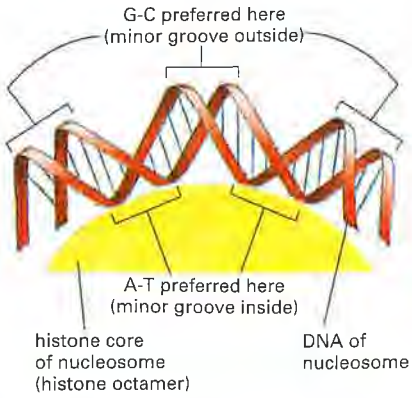


Figure 4–28 The bending of DNA in a nucleosome. The DNA helix makes 1.65 tight turns around the histone octamer. This diagram is drawn approximately to scale, illustrating how the minor groove is compressed on the inside of the turn. Owing to certain structural features of the DNA molecule, A-T base pairs are preferentially accommodated in such a narrow minor groove.

Nucleosomes Are Usually Packed Together into a Compact Chromatin Fiber

Although long strings of nucleosomes form on most chromosomal DNA, chromatin in a living cell probably rarely adopts the extended "beads on a string" form. Instead, the nucleosomes are packed on top of one another, generating regular arrays in which the DNA is even more highly condensed. Thus, when nuclei are very gently lysed onto an electron microscope grid, most of the chromatin is seen to be in the form of a fiber with a diameter of about 30 nm, which is considerably wider than chromatin in the "beads on a string" form (see Figure 4–23).

Several models have been proposed to explain how nucleosomes are packed in the 30-nm chromatin fiber; the one most consistent with the available data is a series of structural variations known collectively as the Zigzag model (Figure 4–29). In reality, the 30-nm structure found in chromosomes is probably a fluid mosaic of the different zigzag variations. We saw earlier that the linker DNA that connects adjacent nucleosomes can vary in length; these differences in linker length probably introduce further local perturbations into the zigzag structure. Finally, the presence of other DNA-binding proteins and DNA sequence that

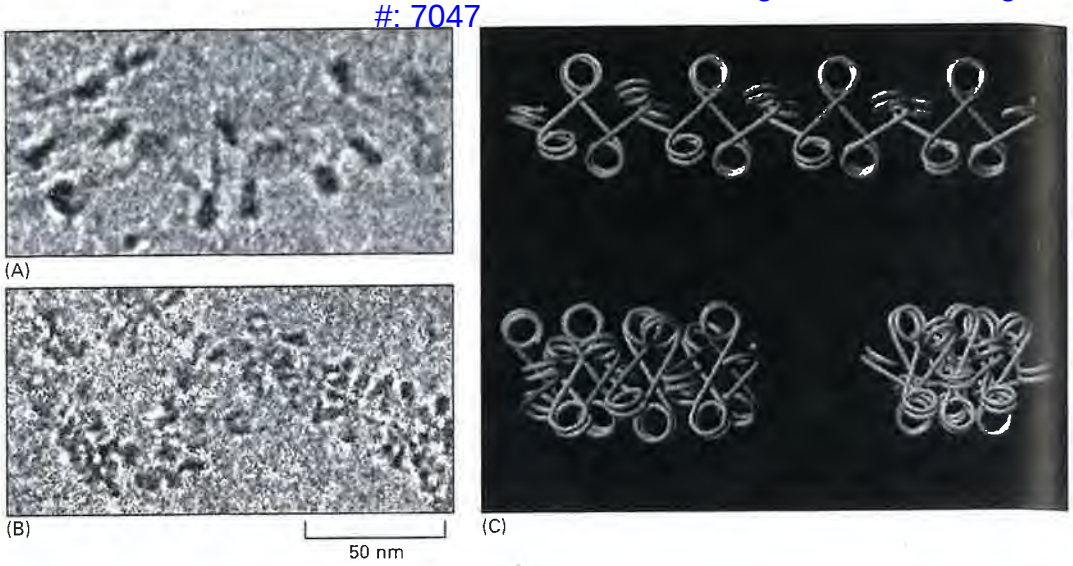


Figure 4–29 Variations on the Zigzag model for the 30-nm chromatin fiber. (A and B) Electron microscopic evidence for the *top* and *bottom-left* model structures depicted in (C). (C) Zigzag variations. An interconversion between these three variations is proposed to occur by an accordion-like expansion and contraction of the fiber length. Differences in the length of the linker between adjacent nucleosome beads can be accommodated by snaking or coiling of the linker DNA, or by small local changes in the width of the fiber. Formation of the 30-nm fiber requires both histone H1 and the core histone tails; for simplicity, neither is shown here, but see Figures 4–30 and 4–32. (From J. Bednar et al., *Proc. Natl. Acad. Sci. USA* 95:14173–14178, 1998. © National Academy of Sciences.)

are difficult to fold into nucleosomes punctuate the 30-nm fiber with irregular features (Figure 4–30).

Several mechanisms probably act together to form the 30-nm fiber from a linear string of nucleosomes. First, an additional histone, called histone H1, is involved in this process. H1 is larger than the core histones and is considerably less well conserved. In fact, the cells of most eucaryotic organisms make several histone H1 proteins of related but quite distinct amino acid sequences. A single histone H1 molecule binds to each nucleosome, contacting both DNA and protein, and changing the path of the DNA as it exits from the nucleosome. Although it is not understood in detail how H1 pulls nucleosomes together into the 30-nm fiber, a change in the exit path in DNA seems crucial for compacting nucleosomal DNA so that it interlocks to form the 30-nm fiber (Figure 4–31).

A second mechanism for forming the 30-nm fiber probably involves the tails of the core histones, which, as we saw above, extend from the nucleosome. It is thought that these tails may help attach one nucleosome to another—thereby allowing a string of them, with the aid of histone H1, to condense into the 30-nm fiber (Figure 4–32).

ATP-driven Chromatin Remodeling Machines Change Nucleosome Structure

For many years biologists thought that, once formed in a particular position on DNA, a nucleosome remained fixed in place because of the tight association

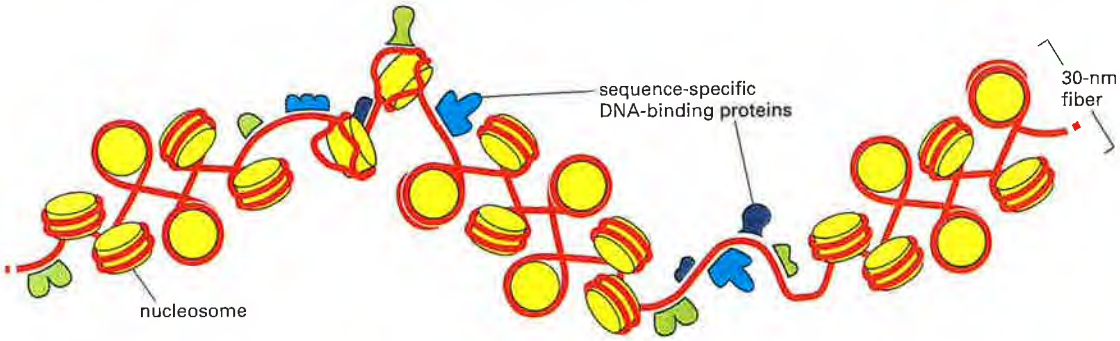


Figure 4–30 Irregularities in the 30-nm fiber. This schematic view of the 30-nm fiber illustrates its interruption by sequence-specific DNA-binding proteins. How these proteins bind tightly to DNA is explained in Chapter 7. The interruptions in the 30-nm fiber may be due to regions of DNA that lack nucleosomes altogether or, more probably, to regions that contain altered or remodeled nucleosomes. Regions of chromatin that are nucleosome free or contain remodeled nucleosome can often be detected experimentally by the unusually high susceptibility of their DNA to digestion by nucleases—as compared with the DNA in nucleosomes.

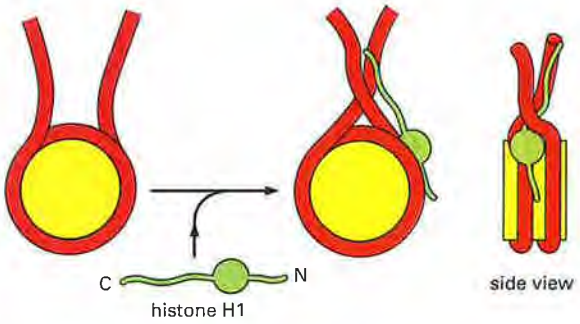


Figure 4–31 A speculative model for how histone H1 could change the path of DNA as it exits from the nucleosome. Histone H1 *(green)* consists of a globular core and two extended tails. Part of the effect of H1 on the compaction of nucleosome organization may result from charge neutralization: like the core histones, H1 is positively charged (especially its C-terminal tail), and this helps to compact the negatively charged DNA. Unlike the core histones, H1 does not seem to be essential for cell viability; in one ciliated protozoan the nucleus expands nearly twofold in the absence of H1, but the cells otherwise appear normal.

between the core histones and DNA. But it has recently been discovered that eucaryotic cells contain *chromatin remodeling complexes*, protein machines that use the energy of ATP hydrolysis to change the structure of nucleosomes temporarily so that DNA becomes less tightly bound to the histone core. The remodeled state may result from movement of the H2A–H2B dimers in the nucleosome core; the H3–H4 tetramer is particularly stable and would be difficult to rearrange (see Figure 4–27).

The remodeling of nucleosome structure has two important consequences. First, it permits ready access to nucleosomal DNA by other proteins in the cell, particularly those involved in gene expression, DNA replication, and repair. Even after the remodeling complex has dissociated, the nucleosome can remain in a "remodeled state" that contains DNA and the full complement of histones—but one in which the DNA–histone contacts have been loosened; only gradually does this remodeled state revert to that of a standard nucleosome. Second, remodeling complexes can catalyze changes in the positions of nucleosomes along DNA (Figure 4–33); some can even transfer a histone core from one DNA molecule to another.

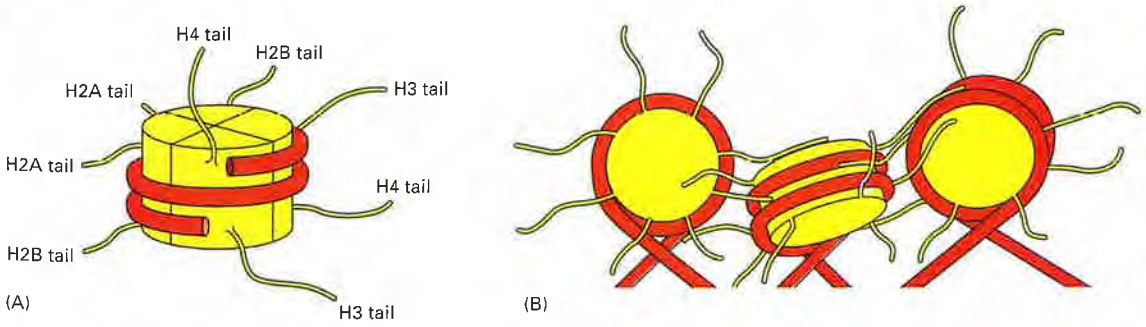
Cells have several different chromatin remodeling complexes that differ subtly in their properties. Most are large protein complexes that can contain more than ten subunits. It is likely that they are used whenever a eucaryotic cell needs direct access to nucleosome DNA for gene expression, DNA replication, or DNA repair. Different remodeling complexes may have features specialized for each of these roles. It is thought that the primary role of some remodeling complexes is to allow access to nucleosomal DNA, whereas that of others is to re-form nucleosomes when access to DNA is no longer required (Figure 4–34).

Chromatin remodeling complexes are carefully controlled by the cell. We shall see in Chapter 7 that, when genes are turned on and off, these complexes can be brought to specific regions of DNA where they act locally to influence chromatin structure. During mitosis, at least some of the chromatin-remodeling complexes are inactivated by phosphorylation. This may help the tightly packaged mitotic chromosomes maintain their structure.

Covalent Modification of the Histone Tails Can Profoundly Affect Chromatin

The N-terminal tails of each of the four core histones are highly conserved in their sequence, and perform crucial functions in regulating chromatin structure.

Figure 4–32 A speculative model for histone tails in the formation of the 30-nm fiber. (A) The approximate exit points of the eight histone tails, four from each histone subunit, that extend from each nucleosome. In the high-resolution structure of the nucleosome (see Figure 4–25), the tails are largely unstructured, suggesting that they are highly flexible. (B) A speculative model showing how the histone tails may help to pack nucleosomes together into the 30-nm fiber. This model is based on (1) experimental evidence that histone tails aid in the formation of the 30-nm fiber, (2) the x-ray crystal structure of the nucleosome, which showed that the tails of one nucleosome contact the histone core of an adjacent nucleosome in the crystal lattice, and (3) evidence that the histone tails interact with DNA.



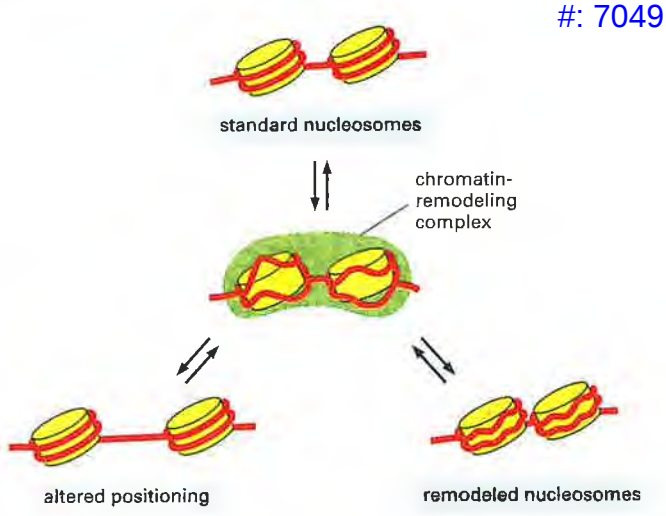


Figure 4–33 Model for the mechanism of some chromatin remodeling complexes. In the absence of remodeling complexes, the interconversion between the three nucleosomal states shown is very slow because of a high activation energy barrier. Using ATP hydrolysis, chromatin-remodeling complexes *(green)* create an activated intermediate (shown in the center of the figure) in which the histone–DNA contacts have been partly disrupted. This activated state can then decay to any one of the three nucleosomal configurations shown. In this way, the remodeling complexes greatly increase the rate of interconversion between different nucleosomal states. The remodeled state, in which the histone–DNA contacts have been loosened, has a higher free energy level than that of standard nucleosomes and will slowly revert to the standard nucleosome conformation, even in the absence of a remodeling complex. Cells have many different chromatin remodeling complexes, and they differ in their detailed biochemical properties; for example, not all can change the position of a nucleosome, but all use the energy of ATP hydrolysis to alter nucleosome structure. (Adapted from R.E. Kingston and G.J. Narlikar, *Genes Dev.* 13:2339–2352, 1999.)

Each tail is subject to several types of covalent modifications, including acetylation of lysines, methylation of lysines, and phosphorylation of serines (Figure 4–35A). Histones are synthesized in the cytosol and then assembled into nucleosomes. Some of the modifications of histone tails occur just after their synthesis, but before their assembly. The modifications that concern us, however, take place once the nucleosome has been assembled. These nucleosome modifications are added and removed by enzymes that reside in the nucleus; for example, acetyl groups are added to the histone tails by histone acetyl transferases (HATs) and taken off by histone deacetylases (HDACs).

The various modifications of the histone tails have several important consequences. Although modifications of the tails have little direct effect on the stability of an individual nucleosome, they seem to affect the stability of the

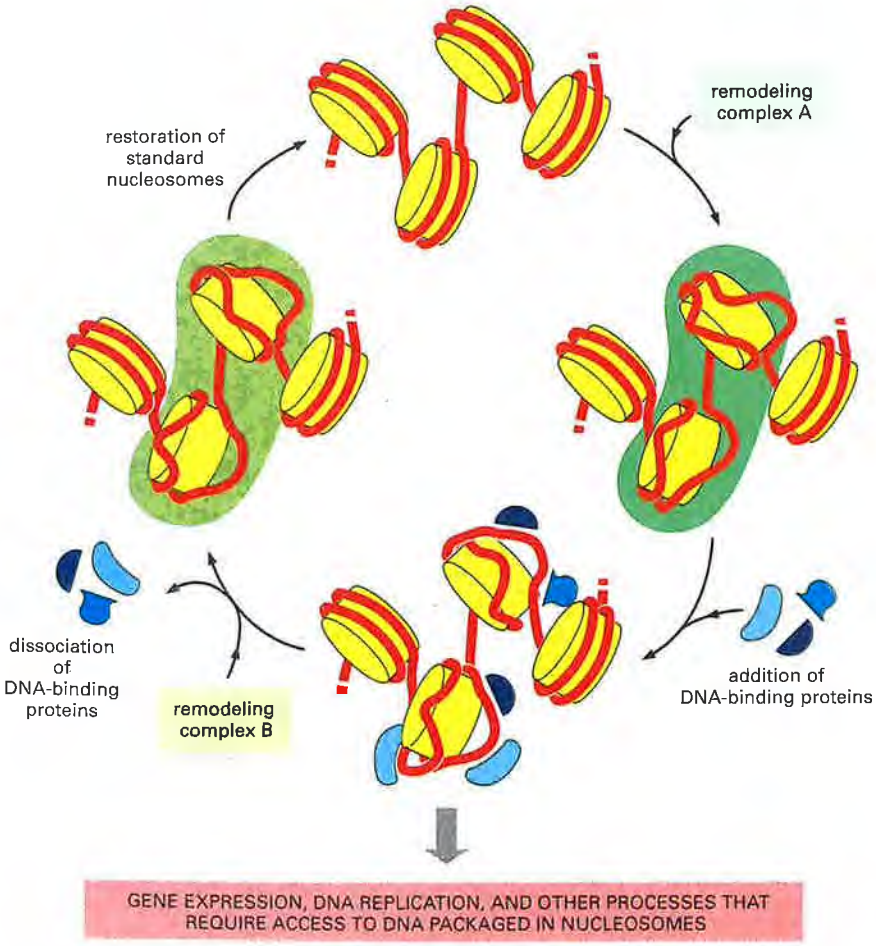
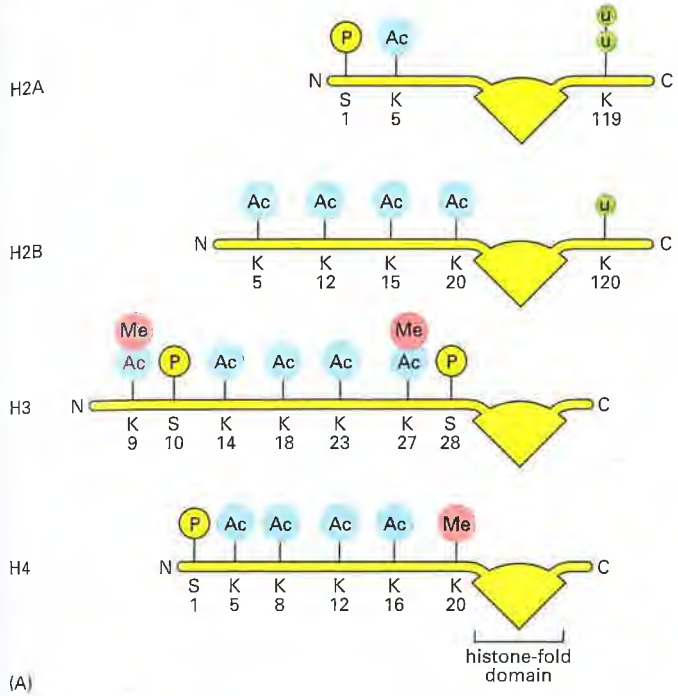


Figure 4–34 A cyclic mechanism for nucleosome disruption and re-formation. According to this model, different chromatin remodeling complexes disrupt and re-form nucleosomes, although, in principle, the same complex might catalyze both reactions. The DNA-binding proteins could function in gene expression, DNA replication, or DNA repair, and in some cases their binding could lead to the dissociation of the histone core to form nucleosome-free regions of DNA like those illustrated in Figure 4–30. (Adapted from A. Travers, *Cell* 96:311–314, 1999.)

30-nm chromatin fiber and of the higher-order structures discussed below. For example, histone acetylation tends to destabilize chromatin structure, perhaps in part because adding an acetyl group removes the positive charge from the lysine, thereby making it more difficult for histones to neutralize the charges on DNA as chromatin is compacted. However, the most profound effect of modified histone tails is their ability to attract specific proteins to a stretch of chromatin that has been appropriately modified. Depending on the precise tail modifications, these additional proteins can either cause further compaction of the chromatin or can facilitate access to the DNA. If combinations of modifications are taken into account, the number of possible distinct markings for each histone tail is very large. Thus, it has been proposed that, through covalent modification of the histone tails, a given stretch of chromatin can convey a particular meaning to



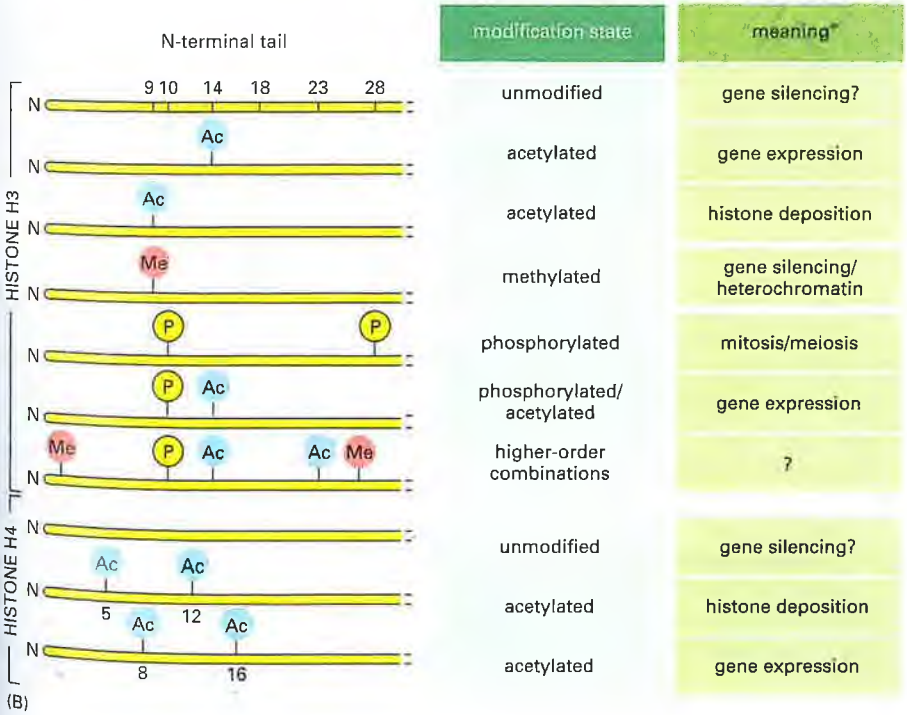


Figure 4–35 Covalent modification of core histone tails. (A) Known modifications of the four histone core proteins are indicated: Me = methyl group, Ac = acetyl group, P = phosphate, u = ubiquitin. Note that some positions (e.g., lysine 9 of H3) can be modified in more than one way. Most of these modifications add a relatively small molecule onto the histone tails; the exception is ubiquitin, a 76 amino acid protein also used in other cellular processes (see Figure 6–87). The function of ubiquitin in chromatin is not well understood: histone H2B can be modified by a single ubiquitin molecule; H2A can be modified by the addition of several ubiquitins. (B) A histone code hypothesis. Histone tails can be marked by different combinations of modifications. According to this hypothesis, each marking conveys a specific meaning to the stretch of chromatin on which it occurs. Only a few of the meanings of the modifications are known. In Chapter 7, we discuss the way a doubly-acetylated H4 tail is "read" by a protein required for gene expression. In another well-studied case, an H3 tail methylated at lysine 9 is recognized by a set of proteins that create an especially compact form of chromatin, which silences gene expression.

The acetylation of lysine 14 of histone H3 and lysines 8 and 16 of histone H4—usually associated with gene expression—is performed by the type A histone acetylases (HATs) in the nucleus. In contrast, the acetylation of lysines 5 and 12 of histone H4 and a lysine of histone H3 takes place in the cytosol, after the histones have been synthesized but before they have been incorporated into nucleosomes; these modifications are catalyzed by type B HATs. These modified histones are deposited onto DNA after DNA replication (see Figure 5–41), and their acetyl groups are taken off shortly afterwards by histone deacetylases (HDACs). Thus, the acetylation at these positions signals newly replicated chromatin.

Modification of a particular position in a histone tail can take on different meanings depending on other features of the local chromatin structure. For example, the phosphorylation of position 10 of histone H3 is associated not only with the condensation of chromosomes that takes place in mitosis and meiosis but also with the expression of certain genes. Some histone tail modifications are interdependent. For example methylation of H3 position 9 blocks the phosphorylation of H3 position 10, and vice versa.

the cell (Figure 4–35B). For example, one type of marking could signal that the stretch of chromatin has been newly replicated, and another could signal that gene expression should not take place. According to this idea, each different marking would attract those proteins that would then execute the appropriate functions. Because the histone tails are extended, and are therefore probably accessible even when chromatin is condensed, they provide an especially apt format for such messages.

As with chromatin remodeling complexes, the enzymes that modify (and remove modifications from) histone tails are usually multisubunit proteins, and they are tightly regulated. They are brought to a particular region of chromatin by other cues, particularly by sequence-specific DNA-binding proteins. We can thus imagine how cycles of histone tail modification and demodification can allow chromatin structure to be dynamic—locally compacting and decompacting it, and, in addition, attracting other proteins specific for each modification state. It is likely that histone-modifying enzymes and chromatin remodeling complexes work in concert to condense and recondense stretches of chromatin; for example, evidence suggests that a particular modification of the histone tail attracts a particular type of remodeling complex. Moreover, some chromatin remodeling complexes contain histone modification enzymes as subunits, directly connecting the two processes.

Summary

A gene is a nucleotide sequence in a DNA molecule that acts as a functional unit for the production of a protein, a structural RNA, or a catalytic RNA molecule. In eucaryotes, protein-coding genes are usually composed of a string of alternating introns and exons. A chromosome is formed from a single, enormously long DNA molecule that contains a linear array of many genes. The human genome contains 3.2×10^9 DNA nucleotide pairs, divided between 22 different autosomes and 2 sex chromosomes. Only a small percentage of this DNA codes for proteins or structural and catalytic RNAs. A chromosomal DNA molecule also contains three other types of functionally important nucleotide sequences: replication origins and telomeres allow the DNA molecule to be completely replicated, while a centromere attaches the daughter DNA molecules to the mitotic spindle, ensuring their accurate segregation to daughter cells during the M phase of the cell cycle.

The DNA in eucaryotes is tightly bound to an equal mass of histones, which form a repeating array of DNA–protein particles called nucleosomes. The nucleosome is composed of an octameric core of histone proteins around which the DNA double helix is wrapped. Despite irregularities in the positioning of nucleosomes along DNA, nucleosomes are usually packed together (with the aid of histone H1 molecules) into quasi-regular arrays to form a 30-nm fiber. Despite the high degree of compaction in chromatin, its structure must be highly dynamic to allow the cell access to the DNA. Two general strategies for reversibly changing local chromatin structures are important for this purpose: ATP-driven chromatin remodeling complexes, and an enzymatically catalyzed covalent modification of the N-terminal tails of the four core histones.

THE GLOBAL STRUCTURE OF CHROMOSOMES

Having discussed the DNA and protein molecules from which the 30-nm chromatin fiber is made, we now turn to the organization of the chromosome on a more global scale. As a 30-nm fiber, the typical human chromosome would still be 0.1 cm in length and able to span the nucleus more than 100 times. Clearly, there must be a still higher level of folding, even in interphase chromosomes. This higher-order packaging is one of the most fascinating—but also one of the most poorly understood—aspects of chromosome structure. Although its molecular basis is still largely a mystery, it almost certainly involves the folding of the 30-nm fiber into a series of loops and coils, as we see below. Our discussion of this higher-order packing continues an important theme in chromosome

architecture: interphase chromatin structure is fluid, exposing at any given moment the DNA sequences directly needed by the cell.

We first describe several rare cases in which the overall structure and organization of interphase chromosomes can be easily visualized, and we explain that certain features of these exceptional cases may be representative of the structures of all interphase chromosomes. Next we describe the different forms of chromatin that make up a typical interphase chromosome. Finally we discuss the additional compaction that interphase chromosomes undergo during the process of mitosis.

Lampbrush Chromosomes Contain Loops of Decondensed Chromatin

Most chromosomes in interphase cells are too fine and too tangled to be visualized clearly. In a few exceptional cases, however, *interphase chromosomes* can be seen to have a precisely defined higher-order structure, and it is thought that certain characteristics of these higher-order structures are representative of all interphase chromosomes. The meiotically paired chromosomes in growing amphibian oocytes (immature eggs), for example, are highly active in gene expression, and they form unusually stiff and extended chromatin loops. These so-called **lampbrush chromosomes** (the largest chromosomes known) are clearly visible even in the light microscope, where they are seen to be organized into a series of large chromatin loops emanating from a linear chromosomal axis (Figure 4–36).

The organization of a lampbrush chromosome is illustrated in Figure 4–37. A given loop always contains the same DNA sequence, and it remains extended in the same manner as the oocyte grows. Other experiments demonstrate that

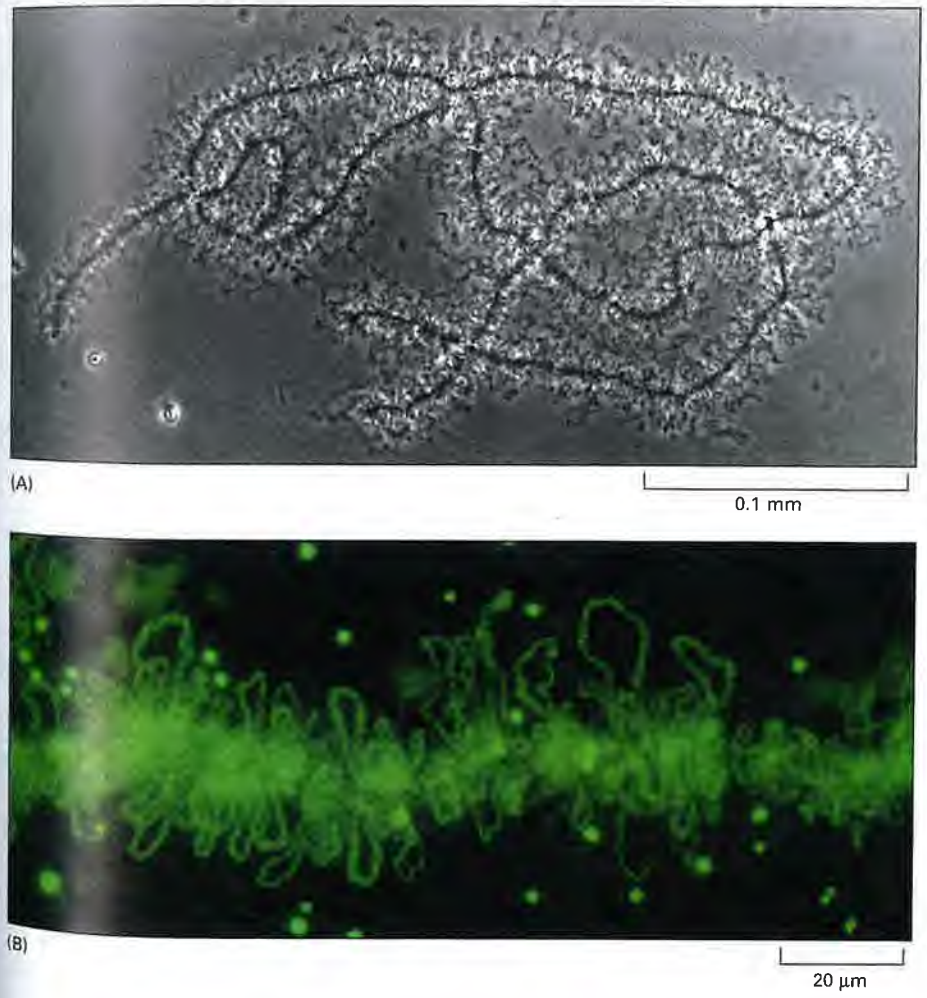


Figure 4–36 Lampbrush chromosomes. (A) A light micrograph of lampbrush chromosomes in an amphibian oocyte. Early in oocyte differentiation, each chromosome replicates to begin meiosis, and the homologous replicated chromosomes pair to form this highly extended structure containing a total of four replicated DNA molecules, or chromatids. The lampbrush chromosome stage persists for months or years, while the oocyte builds up a supply of materials required for its ultimate development into a new individual. (B) Fluorescence light micrograph showing a portion of an amphibian lampbrush chromosome. The regions of the chromosome that are being actively expressed are stained *green* by using antibodies against proteins that process RNA during one of the steps of gene expression (discussed in Chapter 6). The round granules are thought to correspond to large complexes of the RNA-splicing machinery that will also be discussed in Chapter 6. (A, courtesy of Joseph G. Gall; B, courtesy of Joseph G. Gall and Christine Murphy.)

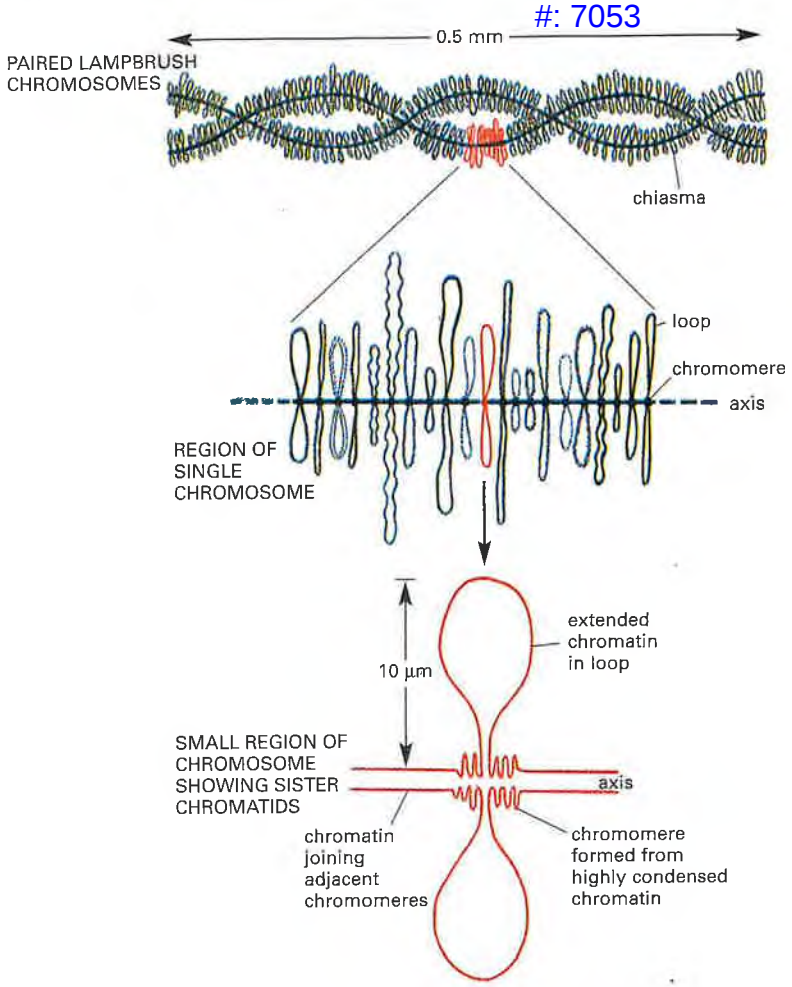


Figure 4–37 A model for the structure of a lampbrush chromosome. The set of lampbrush chromosomes in many amphibians contains a total of about 10,000 chromatin loops, although most of the DNA in each chromosome remains highly condensed in the chromomeres. Each loop corresponds to a particular DNA sequence. Four copies of each loop are present in each cell, since each of the two chromosomes shown at the top consists of two closely apposed, newly replicated chromosomes. This four-stranded structure is characteristic of this stage of development of the oocyte, the diplotene stage of meiosis; see Figure 20–12.

most of the genes present in the DNA loops are being actively expressed (see Figure 4–36B). Most of the DNA, however, is not in loops but remains highly condensed in the *chromomeres* on the axis, which are generally not expressed. Lampbrush chromosomes illustrate a recurrent theme of this chapter—when the DNA in a region of chromatin is in use (in this case, for gene expression), that part of the chromatin has an extended structure; otherwise, the chromatin is condensed. In lampbrush chromosomes, the structural units of this regulation are large, precisely defined loops.

Relatively few species undergo the specialization that produces lampbrush chromosomes. However, when injected into amphibian oocytes, the DNA from organisms that normally do not produce lampbrush chromosomes (e.g., DNA from a fish) is packaged into lampbrush chromosomes. On the basis of this type of experiment, it has been proposed that the interphase chromosomes of all eucaryotes are arranged in loops that are normally too small and fragile to be easily observed. It may be possible in the future to coax the DNA from a mammal such as a mouse to form lampbrush chromosomes by introducing it into amphibian oocytes. This could allow a detailed correlation of loop structure, gene arrangement, and DNA sequence, and we could begin to learn how the packaging into loops reflects the sequence content of our DNA.

Drosophila Polytene Chromosomes Are Arranged in Alternating Bands and Interbands

Certain insect cells also have specialized interphase chromosomes that are readily visible, although this type of specialization differs from that of lampbrush chromosomes. For example, many of the cells of certain fly larvae grow to an enormous size through multiple cycles of DNA synthesis without cell division. The resulting giant cells contain as much as several thousand times the normal DNA complement. Cells with more than the normal DNA complement are said

to be *polyploid* when they contain increased numbers of standard chromosomes. In several types of secretory cells of fly larvae, however, all the homologous chromosome copies are held side by side, like drinking straws in a box, creating a single **polytene chromosome**. The fact that, in some large insect cells, polytene chromosomes can disperse to form a conventional polyploid cell demonstrates that these two chromosomal states are closely related, and that the basic structure of a polytene chromosome must be similar to that of a normal chromosome.

Polytene chromosomes are often easy to see in the light microscope because they are so large and because the precisely aligned side-by-side adherence of individual chromatin strands greatly elongates the chromosome axis and prevents tangling. Polyteny has been most studied in the salivary gland cells of *Drosophila* larvae, in which the DNA in each of the four *Drosophila* chromosomes has been replicated through 10 cycles without separation of the daughter chromosomes, so that 1024 (2^{10}) identical strands of chromatin are lined up side by side (Figure 4–38).

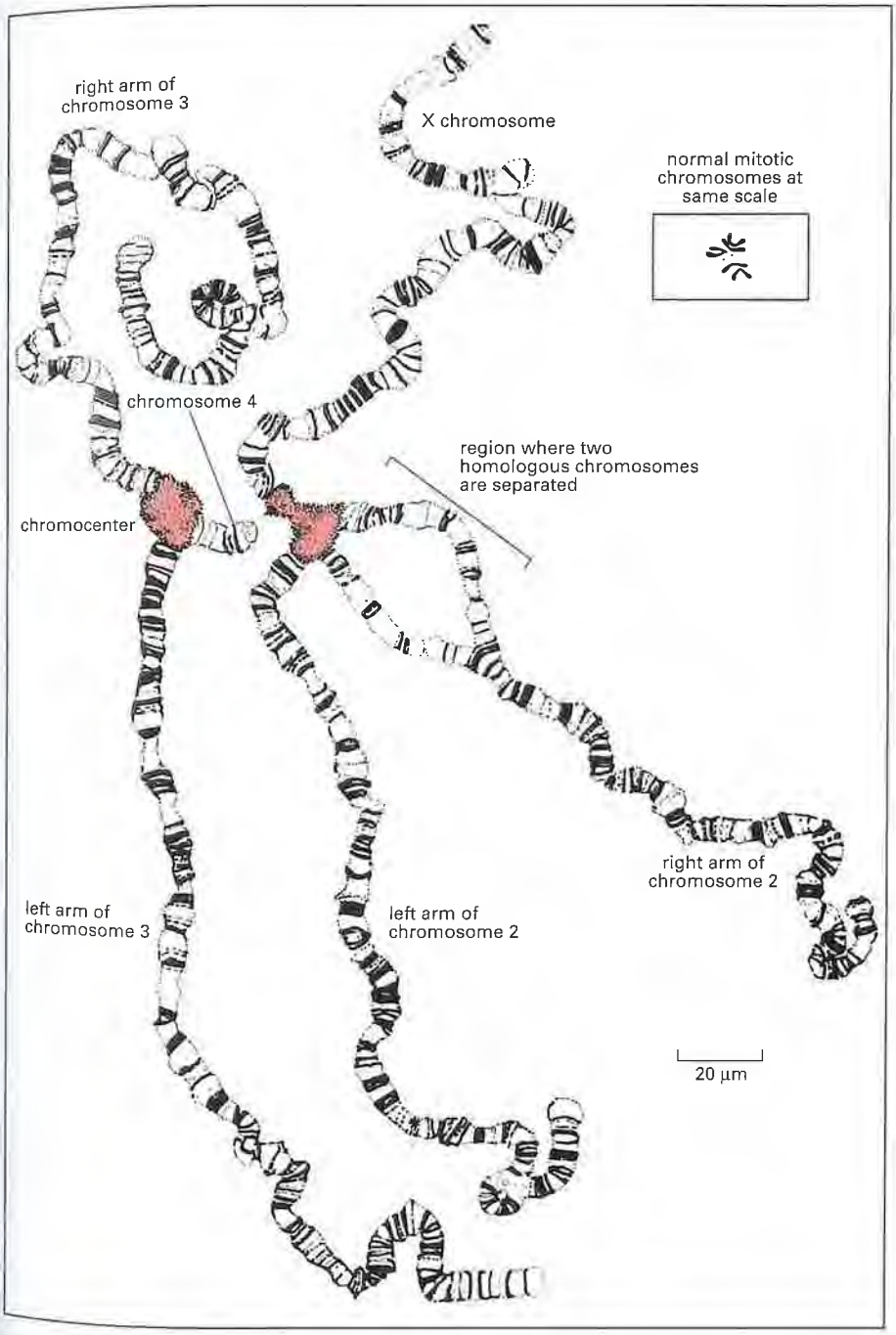


Figure 4–38 The entire set of polytene chromosomes in one *Drosophila* salivary cell. These chromosomes have been spread out for viewing by squashing them against a microscope slide. *Drosophila* has four chromosomes, and there are four different chromosome pairs present. But each chromosome is tightly paired with its homolog (so that each pair appears as a single structure), which is not true in most nuclei (except in meiosis). The four polytene chromosomes are normally linked together by regions near their centromeres that aggregate to create a single large chromocenter *(pink region)*. In this preparation, however, the chromocenter has been split into two halves by the squashing procedure used. (Modified from T.S. Painter, *J. Hered.* 25:465–476, 1934.)

When polytene chromosomes are viewed in the light microscope, distinct alternating dark *bands* and light *interbands* are visible (Figure 4–39). Each band and interband represents a set of 1024 identical DNA sequences arranged in register. About 95% of the DNA in polytene chromosomes is in bands, and 5% is in interbands. The chromatin in each band appears dark, either because it is much more condensed than the chromatin in the interbands, or because it contains a higher proportion of proteins, or both (Figure 4–40). Depending on their size, individual bands are estimated to contain 3000–300,000 nucleotide pairs in a chromatin strand. The bands of *Drosophila* polytene chromosomes can be recognized by their different thicknesses and spacings, and each one has been given a number to generate a chromosome "map." There are approximately 5000 bands and 5000 interbands in the complete set of *Drosophila* polytene chromosomes.

Both Bands and Interbands in Polytene Chromosomes Contain Genes

The reproducible pattern of bands and interbands seen in *Drosophila* polytene chromosomes means that these interphase chromosomes are highly organized. Since the 1930s, scientists have debated the nature of this organization, and we still do not have a clear answer. Because the number of bands in *Drosophila* chromosomes was once thought to be roughly equal to the number of genes in the genome, it was initially thought that each band might correspond to a single gene; however, we now know this simple idea is incorrect. There are nearly three times more genes in *Drosophila* than chromosome bands, and genes are found in both band and interband regions. Moreover, some bands contain multiple genes, and some bands seem to lack genes altogether.

It seems likely that the band–interband pattern reflects different levels of gene expression and chromatin structure along the chromosome, with genes in the less compact interbands being expressed more highly than those in the more compact bands. In any case, the remarkable appearance of fly polytene chromosomes is thought to reflect the heterogeneous nature of the chromatin compaction found along all interphase chromosomes. In the next section we see how the appearance of a band can change dramatically when the gene or genes within it become highly expressed.

Individual Polytene Chromosome Bands Can Unfold and Refold as a Unit

A major factor controlling gene expression in the polytene chromosomes of *Drosophila* is the insect steroid hormone *ecdysone*, the levels of which rise and fall periodically during larval development. When ecdysone concentrations rise,

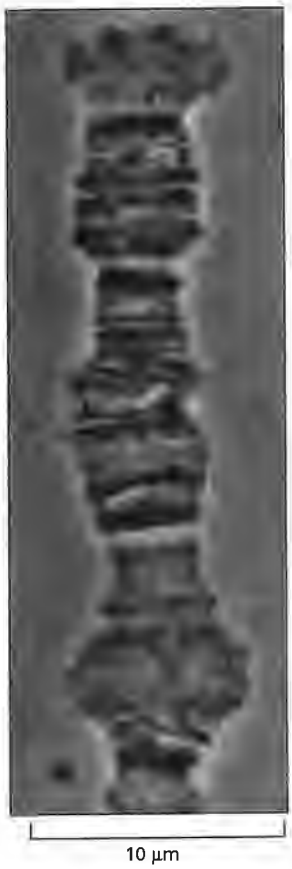


Figure 4–39 A light micrograph of a portion of a polytene chromosome from *Drosophila* salivary glands. The distinct pattern produced by bands and interbands is readily seen. The bands are regions of increased chromatin concentration that occur in interphase chromosomes. Although they are detectable only in polytene chromosomes, it is thought that they reflect a structure common to the chromosomes of most eucaryotes. (Courtesy of Joseph G. Gall.)

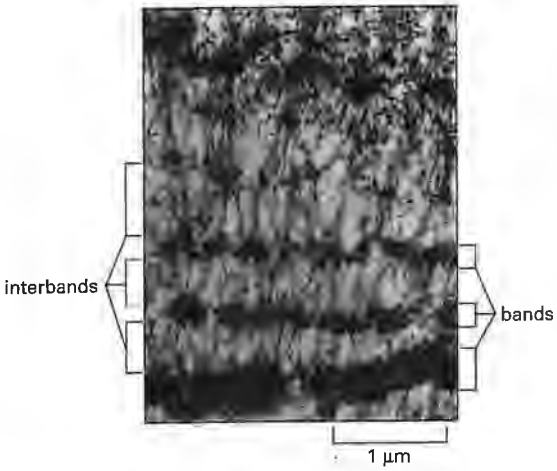


Figure 4–40 An electron micrograph of a small section of a *Drosophila* polytene chromosome seen in thin section. Bands of very different thickness can be readily distinguished, separated by interbands, which contain less condensed chromatin. (Courtesy of Veikko Sorsa.)

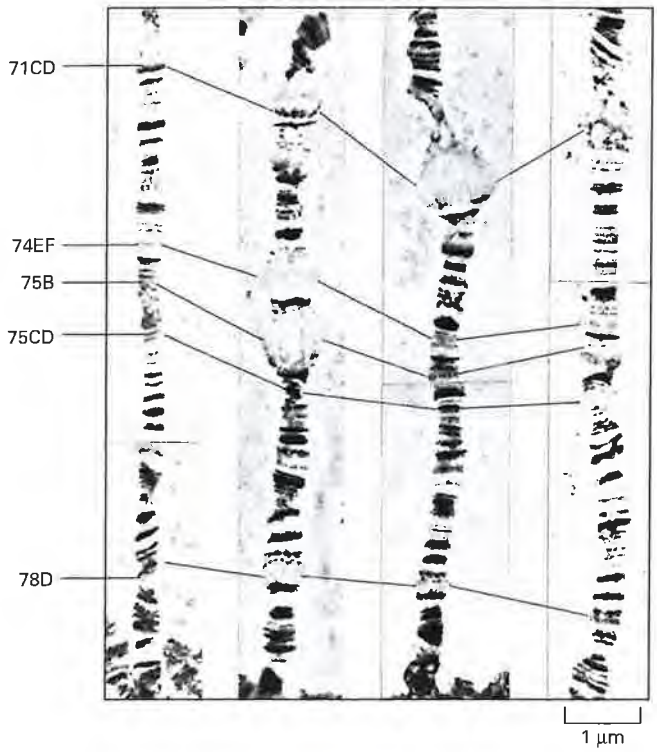
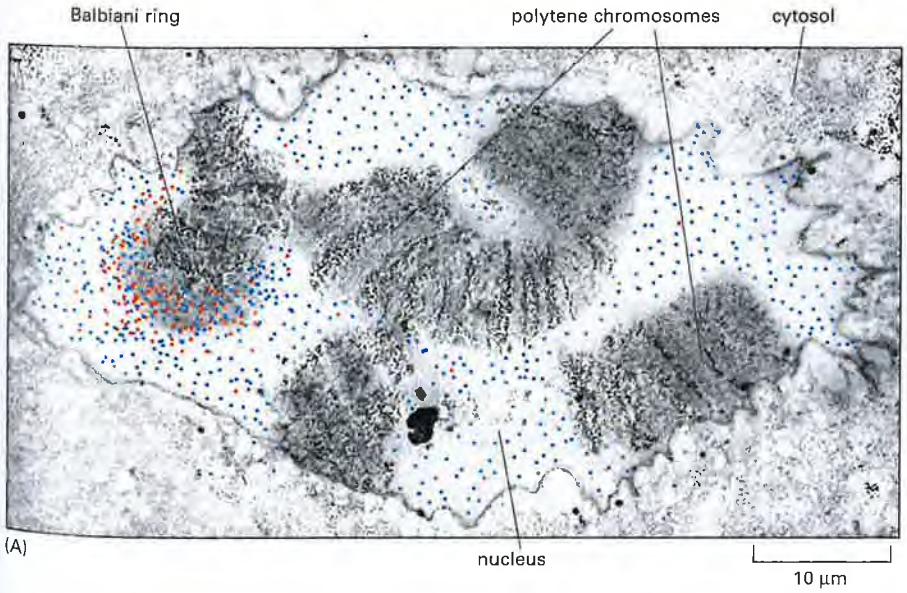


Figure 4–41 Chromosome puffs. This series of time-lapse photographs shows how puffs arise and recede in the polytene chromosomes of *Drosophila* during larval development. A region of the *left* arm of chromosome 3 is shown. It exhibits five very large puffs in salivary gland cells, each active for only a short developmental period. The series of changes shown occur over a period of 22 hours, appearing in a reproducible pattern as the organism develops. The designations of the indicated bands are given at the left of the photographs. (Courtesy of Michael Ashburner.)

they induce the expression of genes coding for proteins that the larva requires for each molt and for pupation. As the organism progresses from one developmental stage to another, distinctive *chromosome puffs* arise and old puffs recede as new genes become expressed and old ones are turned off (Figure 4–41). From inspection of each puff when it is relatively small and the banding pattern is still discernible, it seems that most puffs arise from the decondensation of a single chromosome band.

The individual chromatin fibers that make up a puff can be visualized with an electron microscope. For technical reasons, this is easier in the polytene chromosomes from a different insect, *Chironomus tentans*, a midge. Electron micrographs of certain puffs, called Balbiani rings, of *Chironomus* salivary gland polytene chromosomes show the chromatin arranged in loops (Figures 4–42 and 4–43), much like those observed in the amphibian lampbrush chromosomes discussed earlier. Additional experiments suggest that each loop contains a



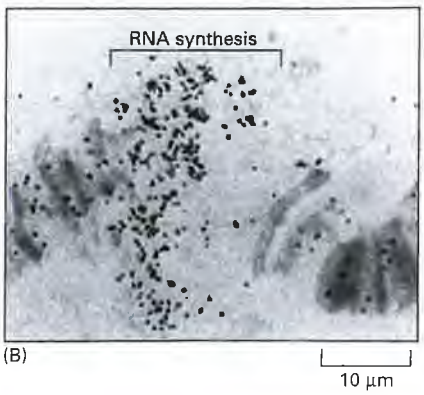


Figure 4–42 RNA synthesis in chromosome puffs. (A) Polytene chromosomes from the salivary glands of the insect *C. tentans*. As outlined in Chapter 1 and described in detail in Chapter 6, the first step in gene expression is the synthesis of an RNA molecule using the DNA as a template. In this electron micrograph, newly synthesized RNA from a Balbiani ring gene is indicated in *red*. Cells were exposed to a brief pulse of BrUTP (an analog of UTP), which was incorporated into RNA. Cells were then fixed and the newly synthesized RNAs were identified by using antibodies against BrU. Balbiani ring RNAs could be distinguished from other RNAs by their characteristic shape (see Figure 4–43). The *blue dots* in the figure represent positions of Balbiani ring RNAs that were synthesized before the addition of BrUTP. The experiment shows that Balbiani ring RNAs are synthesized in puffs and then diffuse through the nucleoplasm. (B) An autoradiogram of a single puff in a polytene chromosome. The portion of the chromosome indicated is undergoing RNA synthesis and has therefore become labeled with ^{3}H-uridine. (A, courtesy of B. Daneholt, from O.P Singh et al., *Exp. Cell Res.* 251:135–146, 1999. © Academic Press; B, courtesy of José Bonner.)

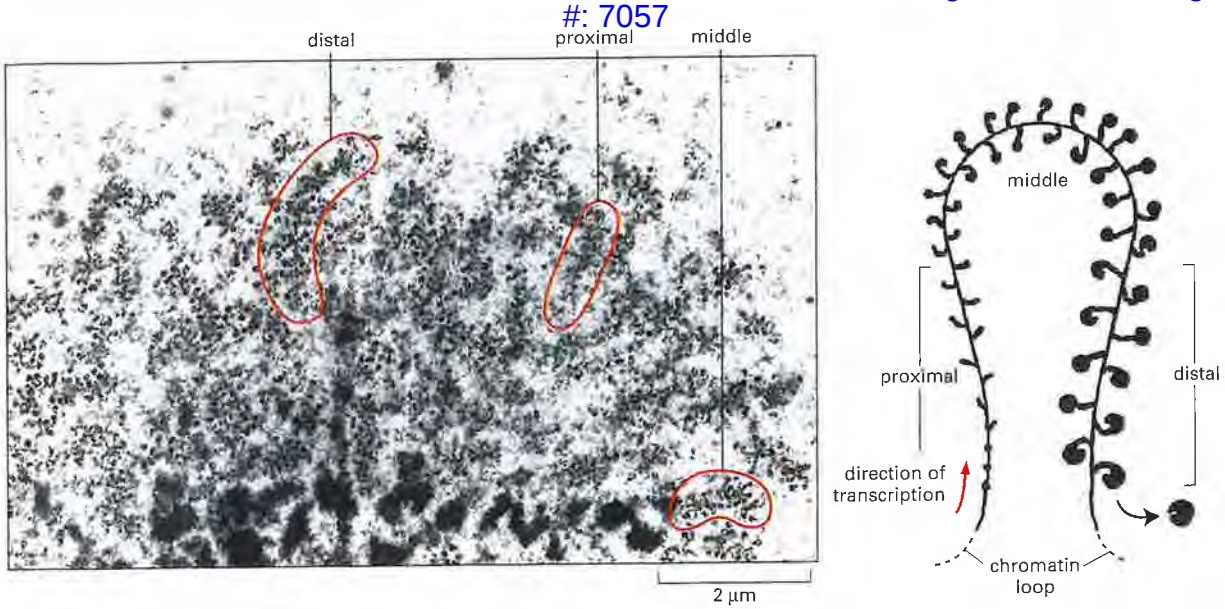


Figure 4–43 Polytene chromosomes from *C. tentans*. The electron micrograph shows a thin section of the chromatin in a Balbiani ring, a chromosome puff very active in gene expression. The Balbiani ring gene codes for secretory proteins the larvae uses to spin a protective tube. The chromatin is arranged in loops, but because the sample has been sectioned, only portions of the loops are visible. As they are synthesized on the chromatin, the RNA molecules are bound up by protein molecules, making them visible as knobs on stalks in the electron microscope. From the size of the RNA–protein complex, the extent of RNA synthesis (transcription) can be inferred; a whole chromatin loop (shown on the right) can then be reconstructed from a set of electron micrograph sections such as that shown here. (Courtesy of B. Daneholt, from U. Skoglund et al., *Cell* 34:847–855, 1983. © Elsevier.)

single gene. When not expressed, the loop of DNA assumes a thickened structure, possibly a folded 30-nm fiber, but when gene expression is occurring, the loop becomes more extended. Both types of loops contain the four core histones and histone H1.

It seems likely that the default loop structure is a folded 30-nm fiber and that the histone modifying enzymes, chromatin remodeling complexes, and other proteins required for gene expression all help to convert it to a more extended form whenever a gene is expressed. In electron micrographs, the chromatin located on either side of the decondensed loop appears considerably more compact, which is consistent with the idea that a loop constitutes an independent functional domain of chromatin structure.

Although controversial, it has been proposed that all of the DNA in polytene chromosomes is arranged in loops that condense and decondense according to when the genes within them are expressed. It may be that all interphase chromosomes from all eucaryotes are also packaged into an orderly series of looped domains, each containing a small number of genes whose expression is regulated in a coordinated way (Figure 4–44). We shall return to this issue in Chapter 7 when we discuss the ways in which gene expression is regulated by the cell.

Heterochromatin Is Highly Organized and Usually Resistant to Gene Expression

Having described some features of interphase chromosomes inferred from a few rare cases, we now turn to characteristics of interphase chromosomes that can be observed in a wide variety of organisms. Light-microscope studies in the 1930s distinguished between two types of chromatin in the interphase nuclei of many higher eucaryotic cells: a highly condensed form, called **heterochromatin**, and all the rest, which is less condensed, called **euchromatin**. Euchromatin is composed of the types of chromosomal structures—30-nm fibers and looped domains—that we have discussed so far. Heterochromatin, in contrast, includes additional proteins and probably represents more compact levels of organization that are just beginning to be understood. In a typical mammalian cell, approximately 10% of the genome is packaged into heterochromatin. Although present in many locations along chromosomes, it is concentrated in specific regions, including the centromeres and telomeres.

Most DNA that is folded into heterochromatin does not contain genes. However, genes that do become packaged into heterochromatin are usually resistant to being expressed, because heterochromatin is unusually compact. This does not mean that heterochromatin is useless or deleterious to the cell; as we see

below, regions of heterochromatin are responsible for the proper functioning of telomeres and centromeres (which lack genes), and its formation may even help protect the genome from being overtaken by "parasitic" mobile elements of DNA. Moreover, a few genes require location in heterochromatin regions if they are to be expressed. In fact, the term *heterochromatin* (which was first defined cytologically) is likely to encompass several distinct types of chromatin structures whose common feature is an especially high degree of organization. Thus, heterochromatin should not be thought of as encapsulating "dead" DNA, but rather as creating different types of compact chromatin with distinct features and roles.

Heterochromatin's resistance to gene expression makes it amenable to study even in organisms in which it cannot be directly observed. When a gene that is normally expressed in euchromatin is experimentally relocated into a region of heterochromatin, it ceases to be expressed, and the gene is said to be *silenced*. These differences in gene expression are examples of **position effects**, in which the activity of a gene depends on its position along a chromosome. First recognized in *Drosophila*, position effects have now been observed in many organisms and they are thought to reflect an influence of the different states of chromatin structure along chromosomes on gene expression. Thus, chromosomes can be considered as mosaics of distinct forms of chromatin, each of which has a special effect on the ability of the DNA it contains to be addressed by the cell.

Many position effects exhibit an additional feature called *position effect variegation*, which is responsible for the mottled appearance of the fly eye and the sectoring of the yeast colony in the examples shown in Figure 4–45. These patterns can result from patches of cells in which a silenced gene has become reactivated; once reactivated, the gene is inherited stably in this form in daughter cells. Alternatively, a gene can start out in euchromatin early in development, and then be selected more or less randomly for packaging into heterochromatin, causing its inactivation in a cell and all of its daughters.

The study of position effect variegation has revealed two important characteristics of heterochromatin. First, heterochromatin is dynamic; it can "spread" into a region and later "retract" from it at low but observable frequencies. Second, the state of chromatin—whether heterochromatin or euchromatin—tends to be inherited from a cell to its progeny. These two features are responsible for position effect variegation, as explained in Figure 4–46. In the next section, we discuss several models to account for the self-sustaining nature of heterochromatin, once it has been formed.

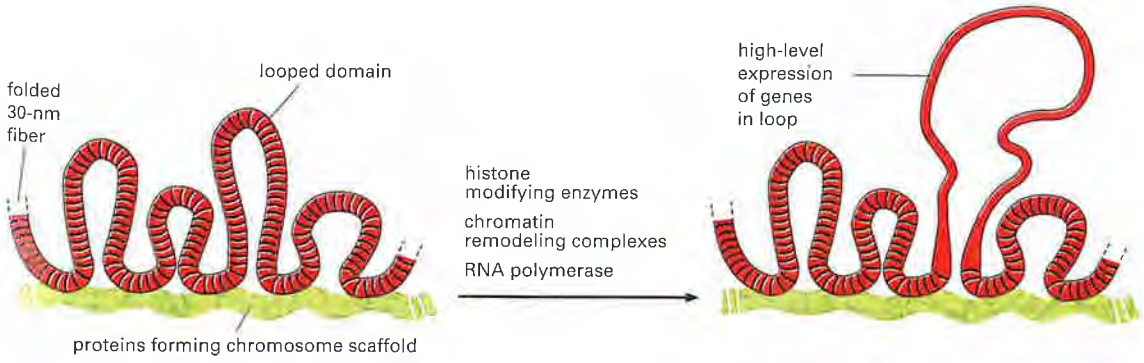


Figure 4–44 A model for the structure of an interphase chromosome. A section of an interphase chromosome is shown folded into a series of looped domains, each containing 20,000–100,000 nucleotide pairs of double-helical DNA condensed into a 30-nm fiber. Individual loops can decondense, perhaps in part through an accordionlike expansion of the 30-nm fiber (see Figure 4–29), when the cell requires direct access to the DNA packaged in these loops. This decondensation is brought about by enzymes that directly modify chromatin structure—as well as by proteins, such as RNA polymerase (discussed in Chapter 6), that act directly on the underlying DNA. It is not understood how the folded 30-nm fiber is anchored to the chromosome axis, but evidence suggests that the base of chromosomal loops is rich in DNA topoisomerases, which are enzymes that allow DNA to swivel when anchored (see pp. 251–253).

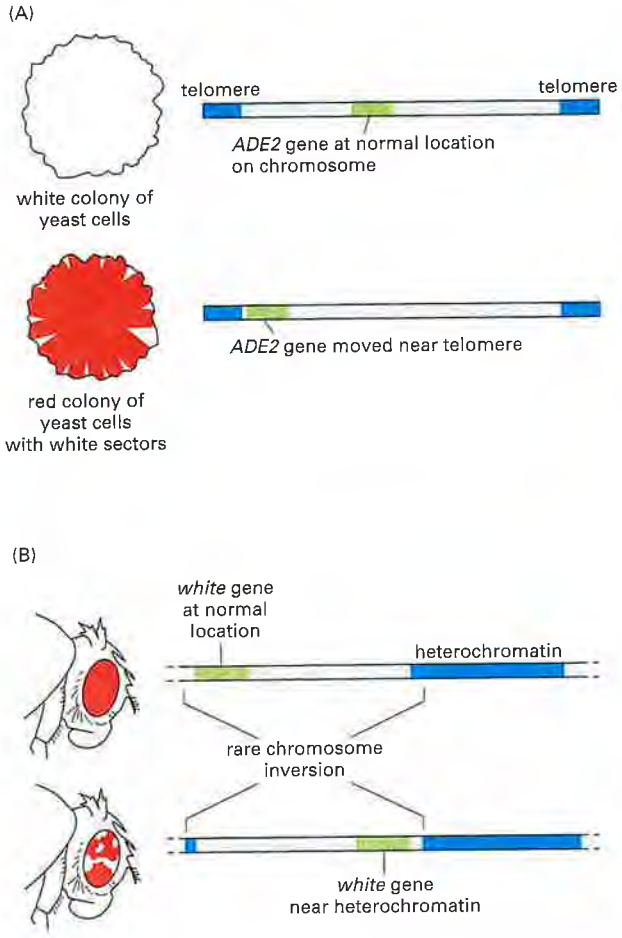


Figure 4–45 Position effects on gene expression in two different eucaryotic organisms. (A) The yeast *ADE2* gene at its normal chromosomal location is expressed in all cells. When moved near the end of a yeast chromosome, which is inferred to be folded into a form of heterochromatin, the gene is no longer expressed in most cells of the population. The *ADE2* gene codes for one of the enzymes of adenine biosynthesis, and the absence of the *ADE2* gene product leads to the accumulation of a red pigment. Therefore, a colony of cells that expresses *ADE2* is *white*, and one composed of cells where the *ADE2* gene is not expressed is *red*. The *white* sectors that fan out from the middle of the *red* colony grown on an agar surface represent the descendants of cells in which the *ADE2* gene has spontaneously become active. These white sectors are thought to result from a heritable change to a less tightly packed state of chromatin near the *ADE2* gene in these cells. Although yeast chromosomes are too small to be seen under the light microscope, the chromatin structure at the ends of yeast chromosomes is thought to have many of the same structural features as the heterochromatin in the chromosomes of larger organisms.

(B) Position effects can also be observed for the *white* gene in the fruit fly *Drosophila*. The *white* gene controls eye pigment production and is named after the mutation that first identified it. Wild-type flies with a normal *white* gene (*white*$^-$) have normal pigment production, which gives them red eyes, but if the *white* gene is mutated and inactivated, the mutant flies (*white*$^-$) make no pigment and have *white* eyes. In flies in which a normal *white*$^+$ gene has been moved near a region of heterochromatin, the eyes are mottled, with both *red* and *white* patches. The *white* patches represent cells in which the *white*$^+$ gene has been silenced by the effects of the heterochromatin. In contrast, the red patches represent cells that express the *white*$^+$ gene because the heterochromatin did not spread across this gene at the time, early in development, when the heterochromatin first formed. As in the yeast, the presence of large patches of *red* and *white* cells indicates that the state of transcriptional activity of the gene is inherited, once determined by its chromatin packaging in the early embryo.

The Ends of Chromosomes Have a Special Form of Heterochromatin

Unlike the nucleosome and the 30-nm fiber, heterochromatin is not well understood structurally. It almost certainly involves an additional level of folding of 30-nm fiber and requires many proteins in addition to the histones. Although its chromosomes are too small to be seen under the light microscope, the molecular nature of heterochromatin is probably best understood in the simple yeast *S. cerevisiae*. Many experiments with yeast cells have shown that the chromatin extending inward roughly 5000 nucleotide pairs from each chromosome end is resistant to gene expression, and probably has a structure that corresponds to at least one type of heterochromatin in the chromosomes of more complex organisms. Extensive genetic analysis has led to the identification of many of the yeast proteins required for this type of gene silencing.

Mutations in any one of a set of yeast *S*ilent *i*nformation *r*egulator (Sir) proteins prevent the silencing of genes located near telomeres, thereby allowing these genes to be expressed. Analysis of these proteins has led to the discovery of a telomere-bound Sir protein complex that recognizes underacetylated N-terminal tails of selected histones (Figure 4–47A). One of the proteins in this complex is a highly conserved histone deacetylase known as Sir2, which has homologs in diverse organisms, including humans, and presumably has a major role in creating a pattern of histone underacetylation unique to heterochromatin. As discussed earlier in this chapter, deacetylation of the histone tails is thought to allow nucleosomes to pack together into tighter arrays and may also render them less susceptible to some chromatin remodeling complexes. In addition, heterochromatin-specific patterns of histone tail modification are likely to attract additional proteins involved in forming and maintaining heterochromatin (see Figure 4–35).

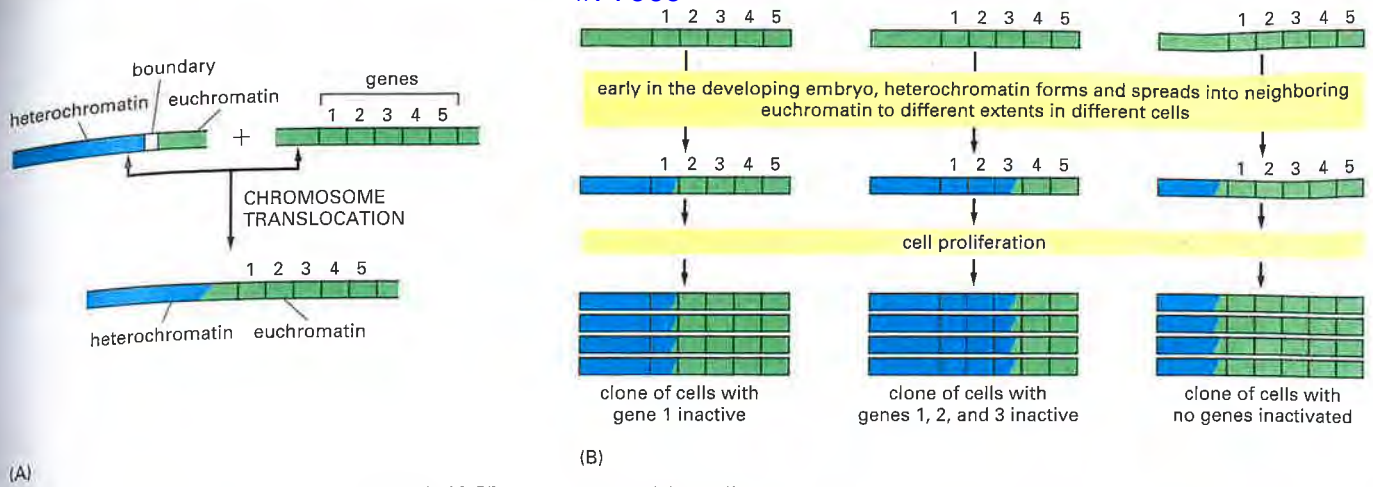


Figure 4–46 The cause of position effect variegation in *Drosophila*. (A) Heterochromatin *(blue)* is normally prevented from spreading into adjacent regions of euchromatin *(green)* by special boundary DNA sequences, which we discuss in Chapter 7. In flies that inherit certain chromosomal rearrangements, however, this barrier is no longer present. (B) During the early development of such flies, heterochromatin can spread into neighboring chromosomal DNA, proceeding for different distances in different cells. This spreading soon stops, but the established pattern of heterochromatin is inherited, so that large clones of progeny cells are produced that have the same neighboring genes condensed into heterochromatin and thereby inactivated (hence the "variegated" appearance of some of these flies; see Figure 4–45B). Although "spreading" is used to describe the formation of new heterochromatin near previously existing heterochromatin, the term may not be wholly accurate. There is evidence that during expansion, heterochromatin can "skip over" some regions of chromatin, sparing the genes that lie within them from repressive effects. One possibility is that heterochromatin can expand across the base of some DNA loops, thus bypassing the chromatin contained in the loop.

But how is the Sir2 protein delivered to the ends of chromosomes in the first place? Another series of experiments has suggested the model shown in Figure 4–47B. A DNA-binding protein that recognizes specific DNA sequences in yeast telomeres also binds to one of the Sir proteins, causing the entire Sir protein complex to assemble on the telomeric DNA. The Sir complex then spreads along the chromosome from this site, modifying the N-terminal tails of adjacent histones

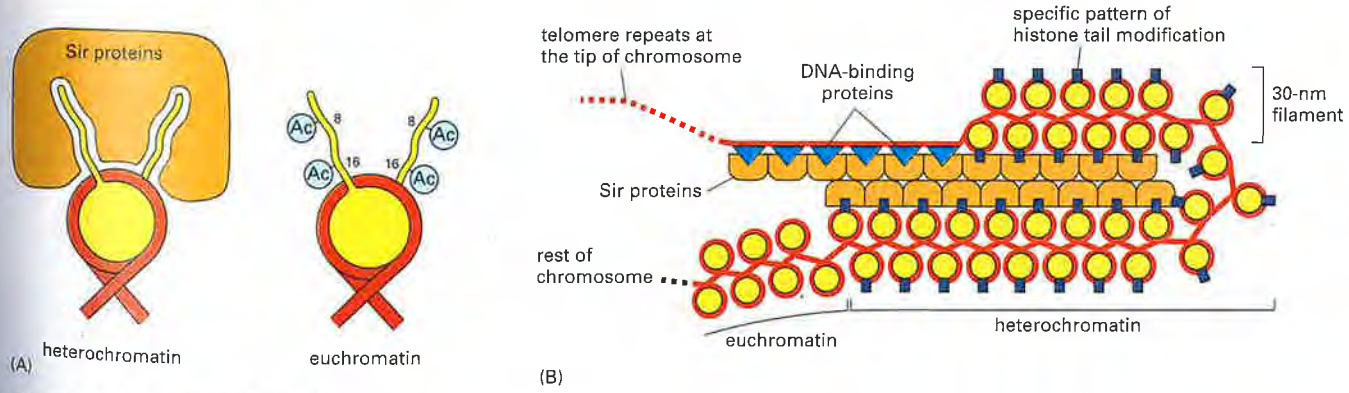


Figure 4–47 Speculative model for the heterochromatin at the ends of yeast chromosomes. (A) Heterochromatin is generally underacetylated, and underacetylated tails of histone H4 are proposed to interact with a complex of Sir proteins, thus stabilizing the association of these proteins with nucleosomes. Although shown as fully unacetylated, the exact pattern of histone H4 tail modification required to bind to the Sir complex is not known with certainty. In some organisms, the methylation of lysine 9 of histone H3 is also a critical signal for heterochromatin formation. In euchromatin, histone tails are typically highly acetylated. Those of H4 are shown as partially acetylated but, in reality, the acetylation state varies across euchromatin. (B) Specialized DNA-binding proteins *(blue triangles)* recognize DNA sequences near the ends of chromosomes and attract the Sir proteins, one of which (Sir2) is an NAD^+-dependent histone deacetylase. This then leads to the cooperative spreading of the Sir protein complex down the chromosome. As this complex spreads, the deacetylation catalyzed by Sir2 helps create new binding sites on nucleosomes for more Sir protein complexes. A "fold back" structure of the type shown may also form.

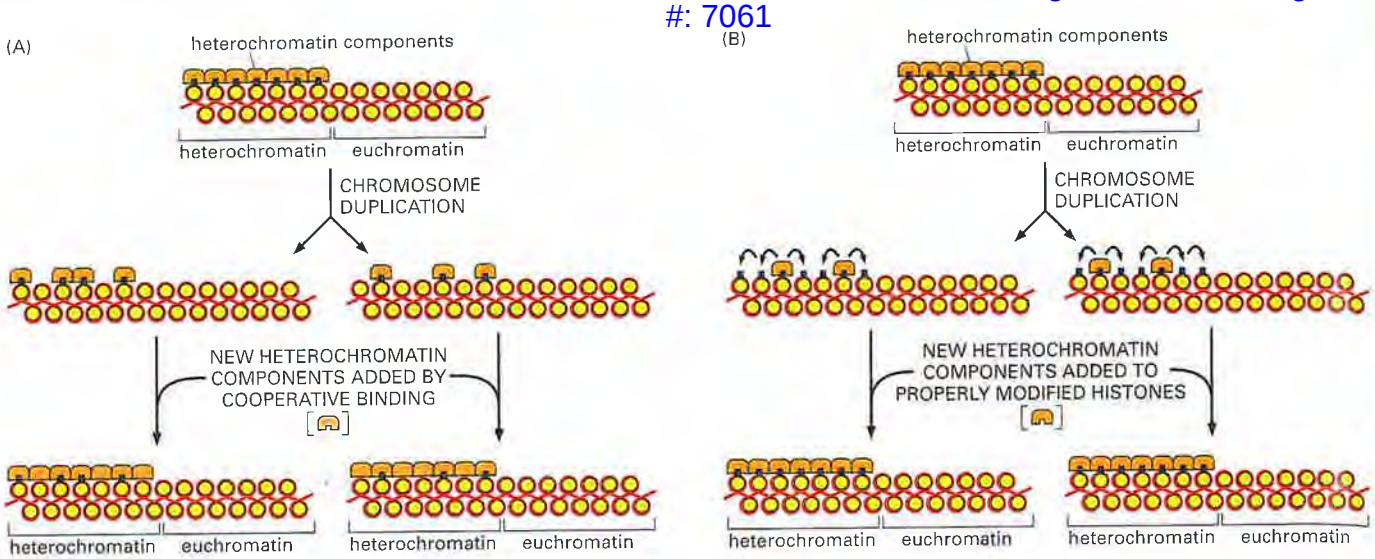


Figure 4–48 Two speculative models for how the tight packaging of DNA in heterochromatin can be inherited during chromosome replication. In both cases, half of the specialized heterochromatin components have been distributed to each daughter chromosome after DNA duplication. (A) In this model, new heterochromatin components bind cooperatively to the inherited components, thereby beginning the process of new heterochromatin formation. The process is completed with the assembly of additional proteins and the eventual covalent modification of the histones (not shown). (B) In this model, the inherited heterochromatin components change the pattern of histone modification on the newly formed daughter nucleosomes nearby, creating new binding sites for free heterochromatin components, which assemble and complete the structure. Both models can account for the spreading effects of heterochromatin, and indeed, both processes may occur simultaneously in cells.

to create the nucleosome-binding sites that the complex prefers. This "spreading effect" is thought to be driven by the cooperative binding of adjacent Sir protein complexes, as well as by the folding back of the chromosome on itself to promote Sir binding in nearby regions (see Figure 4–47B). In addition, the formation of heterochromatin probably requires the action of chromatin remodeling complexes to readjust the positions of nucleosomes as they are packed together.

Unlike most deacetylases, Sir2 requires NAD^+ as a cofactor (see Figure 2–60). The NAD^+ levels in the cell fluctuate with the nutritional health of the cell, increasing as cells become nutritionally deprived. This feature might cause the telomeric heterochromatin to spread in response to starvation (perhaps to silence the expression of genes that are not absolutely required for survival) and then to retract when conditions improve.

The properties of the yeast heterochromatin just described may resemble features of heterochromatin in more complex organisms. Certainly, the spreading of yeast heterochromatin from telomeres is similar in principle to the movement of heterochromatin that causes position effect variegation in animals (see Figure 4–46). Moreover, these properties can be used to explain the heritability of heterochromatin, as outlined in Figure 4–48. Whatever the precise mechanism of heterochromatin formation, it has become clear that covalent modifications of the nucleosome core histones have a critical role in this process. Of special importance in many organisms are the *histone methyl transferases,* enzymes that methylate specific lysines on histones including lysine 9 of histone H3 (see Figure 4–35). This modification is "read" by heterochromatin components (including HP1 in *Drosophila)* that specifically bind this modified form of histone H3 to induce the assembly of heterochromatin. It is likely that a spectrum of different histone modifications is used by the cell to distinguish heterochromatin from euchromatin (see Figure 4–35).

Having the ends of chromosomes packaged into heterochromatin provides several advantages to the cell: it helps to protect the ends of chromosomes from being recognized as broken chromosomes by the cellular repair machinery, it may help to regulate telomere length, and it may assist in the accurate pairing and segregation of chromosomes during mitosis. In Chapter 5 we see that telomeres have additional structural features that distinguish them from other parts of chromosomes.

Centromeres Are Also Packaged into Heterochromatin

Heterochromatin is also observed around centromeres, the DNA sequences that direct the movement of each chromosome into daughter cells every time a cell divides (see Figure 4–22). In many complex organisms, including humans, each

centromere seems to be embedded in a very large stretch of heterochromatin that persists throughout interphase, even though the centromere-directed movement of DNA occurs only during mitosis. The structure and biochemical properties of this so-called *centric heterochromatin* are not well understood, but, like other forms of heterochromatin, it silences the expression of genes that are experimentally placed into it. It contains, in addition to histones (which are typically underacetylated and methylated in heterochromatin), several additional structural proteins that compact the nucleosomes into particularly dense arrangements.

As with telomeres, our best understanding of the chromatin structure of a centromere comes from studies of the much simpler centromeres of the yeast *S. cerevisiae*. Earlier in this chapter we saw that a simple DNA sequence of approximately 125 nucleotide pairs was sufficient to serve as a centromere in this organism. Despite its small size, more than a dozen different proteins assemble on this DNA sequence; the proteins include a histone H3 variant that, along with the other core histones, is believed to form a centromere-specific nucleosome (Figure 4–49A). We do not yet understand what properties this variant type of nucleosome provides to the cell, but similar specialized nucleosomes seem to be present in all eucaryotic centromeres (Figure 4–49B). The additional proteins at the yeast centromere attach it to the spindle microtubules and provide signals that ensure that this attachment is complete before the later stages of mitosis are allowed to proceed (discussed in Chapters 17 and 18).

The centromeres in more complex organisms are considerably larger than those in budding yeasts. For example, fly and human centromeres extend over hundreds of thousands of nucleotide pairs and do not seem to contain a centromere-specific DNA sequence. Rather, most consist largely of short, repeated DNA sequences, known as *alpha satellite DNA* in humans (Figure 4–50). But the same repeat sequences are also found at other (noncentromeric) positions on chromosomes, and how they specify a centromere is poorly understood. Somehow the formation of the inner plate of a kinetochore is "seeded," followed by the cooperative assembly of the entire group of special proteins that form the kinetochore (Figure 4–50B). It seems that centromeres in complex organisms are defined more by an assembly of proteins than by a specific DNA sequence.

There are some striking similarities between the formation and maintenance of centromeres and the formation and maintenance of other regions of heterochromatin. The entire centromere forms as an all-or-none entity, suggesting a highly cooperative addition of proteins after a seeding event. Moreover, once formed, the structure seems to be directly inherited on the DNA as part of each round of chromosome replication. Thus, for example, some regions of our chromosomes contain nonfunctional alpha satellite DNA sequences that seem to be identical to those at the centromere; these sequences are presumed to have arisen from a chromosome-joining event that initially created one chromosome with two centromeres (an unstable, dicentric

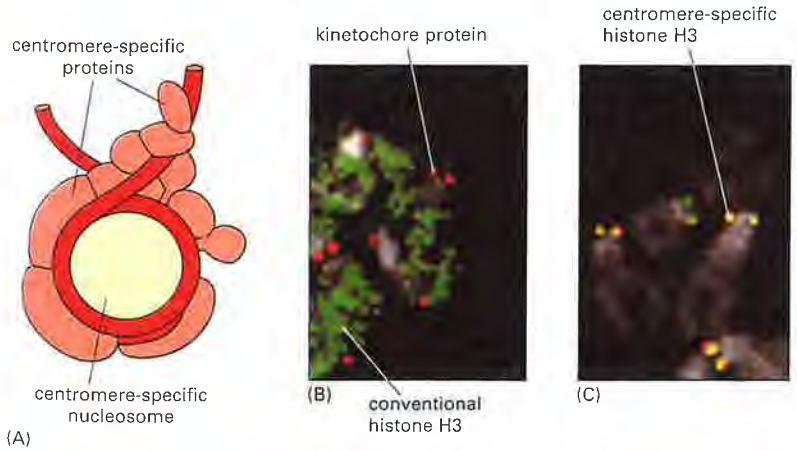


Figure 4–49 The specialized nucleosome formed on centromeres. (A) A model for the proteins that assemble on a yeast centromere. The specialized nucleosome contains an H3 variant (called CENP-A in most organisms), along with core histones H2A, H2B, and H4. The folding of DNA into this nucleosome facilitates the assembly of the other centromere-binding proteins, which form the kinetochore that attaches the centromere to the mitotic spindle (B) The localization of conventional histone H3 on *Drosophila* mitotic chromosomes. The conventional H3 has been fused to a fluorescent protein and appears *green*. A component of the kinetochore has been stained *red* with antibodies against a specific kinetochore protein. (C) The same experiment, but with the centromere-specific histone H3 (instead of the conventional H3) labeled *green*. When the *red* and *green* stains are coincident, the staining appears *yellow*. (A, adapted from P.B. Meluh et al., *Cell* 94:607–613, 1998; B and C, from S. Henikoff et al., *Proc. Natl. Acad. Sci. USA* 97:716–721, 2000. © National Academy of Sciences.)

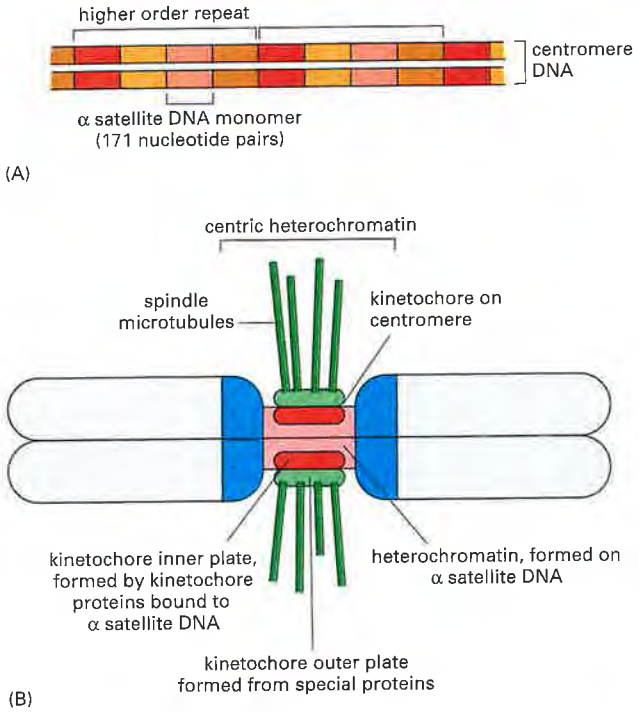


Figure 4–50 The structure of a human centromere. (A) The organization of the alpha satellite DNA sequences, which are repeated many thousands of times at a centromere. (B) An entire chromosome. The alpha satellite DNA sequences *(red)* are AT-rich and consist of a large number of repeats that vary slightly from one another in their DNA sequence. *Blue* represents the position of flanking centric heterochromatin, which contains DNA sequences composed of different types of repeats. As indicated, the kinetochore consists of an inner and an outer plate, formed by a set of kinetochore proteins. The spindle microtubules attach to the kinetochore in M phase of the cell cycle (see Figure 4–22). (B, adapted from T.D. Murphy and G.H. Karpen, *Cell* 93:317–320, 1998.)

chromosome; Figure 4–51A). Moreover, in some unusual cases, new human centromeres (called neocentromeres) have been observed to form spontaneously on fragmented chromosomes. Some of these new positions were originally euchromatic and lack alpha satellite DNA altogether (Figure 4–51B).

To explain these observations it has been proposed that *de novo* centromere formation requires an initial marking (or seeding) event, perhaps the formation of a specialized DNA–protein structure, which, in humans, happens more readily on arrays of alpha satellite DNA than on other DNA sequences. This mark would be duplicated when the chromosome divides, and the same centromere would then function in the next cell division. Very rarely, the mark would be lost after chromosome replication, in which case it would be very difficult to establish again (Figure 4–51C). Although the self-renewing nature of centromeres is not understood in detail, the type of models described for heterochromatin inheritance in Figure 4–48 could also be critical here.

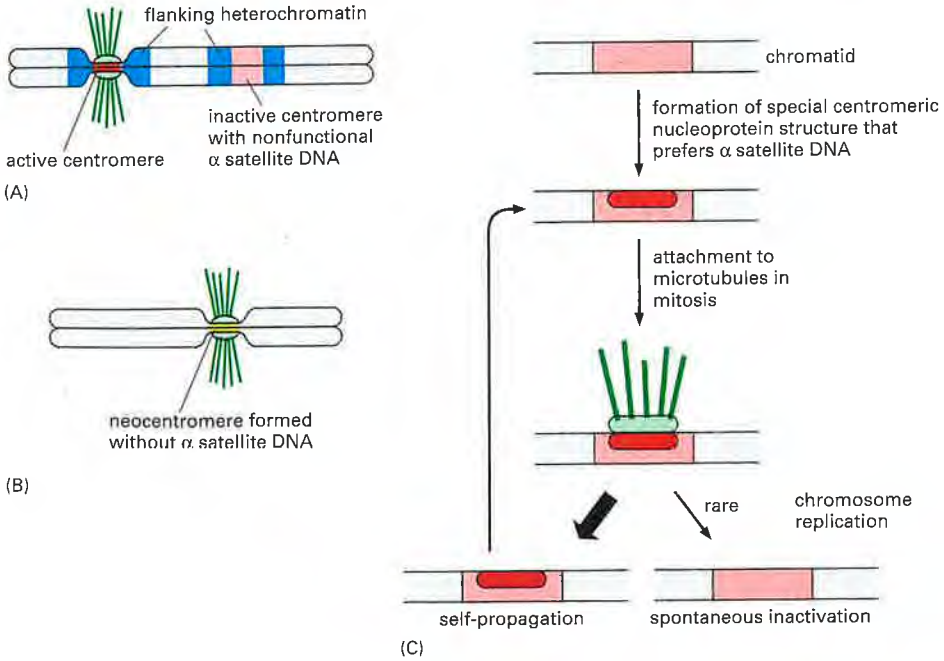


Figure 4–51 The plasticity of human centromere formation. (A) Owing to an ancient chromosome breakage and rejoining event, some human chromosomes contain two blocks of alpha satellite DNA *(red)*, each of which presumably functioned as a centromere in its original chromosome. Usually, these dicentric chromosomes are not stably propagated because they are attached improperly to the spindle and are broken apart during mitosis. In those chromosomes that survive, one of the centromeres has spontaneously inactivated, even though it contains all the necessary DNA sequences. This allows the chromosome to be stably propagated. (B) In a small fraction (1/2000) of human births, extra chromosomes are observed in cells of the offspring. Some of these extra chromosomes, which have formed from a breakage event, lack alpha satellite DNA altogether, yet new centromeres have arisen from what was originally euchromatic DNA. (C) A model to explain the plasticity and inheritance of centromeres.

The plasticity of centromeres may provide an important evolutionary advantage. We have seen that chromosomes evolve in part by breakage and rejoining events (see Figure 4–19). Many of these events produce chromosomes with two centromeres, or chromosome fragments with no centromeres at all. Although rare, the inactivation of centromeres and their ability to be activated *de novo* may occasionally allow newly formed chromosomes to be maintained stably and thereby facilitate the process of chromosome evolution.

Heterochromatin May Provide a Defense Mechanism Against Mobile DNA Elements

DNA packaged in heterochromatin often consists of large tandem arrays of short, repeated sequences that do not code for protein, as we saw above for the heterochromatin of mammalian centromeres. In contrast, euchromatic DNA is rich in genes and other single-copy DNA sequences. Although this correlation is not absolute (some arrays of repeated sequences exist in euchromatin and some genes are present in heterochromatin), this trend suggests that some types of repeated DNA may be a signal for heterochromatin formation. This idea is supported by experiments in which several hundred tandem copies of genes have been artificially introduced into the germ lines of flies and mice. In both organisms these gene arrays are often silenced, and in some cases, they can be observed under the microscope to have formed regions of heterochromatin. In contrast, when single copies of the same genes are introduced into the same position in the chromosome, they are actively expressed.

This feature, called *repeat-induced gene silencing*, may be a mechanism that cells have for protecting their genomes from being overtaken by mobile genetic elements. These elements, which are discussed in Chapter 5, can multiply and insert themselves throughout the genome. According to this idea, once a cluster of such mobile elements has formed, the DNA that contains them would be packaged into heterochromatin to prevent their further proliferation. The same mechanism could be responsible for forming the large regions of heterochromatin that contain large numbers of tandem repeats of a simple sequence, as occurs around centromeres.

Mitotic Chromosomes Are Formed from Chromatin in Its Most Condensed State

Having discussed the dynamic structure of interphase chromosomes, we now turn to the final level of DNA packaging, that observed for mitotic chromosomes. With the exception of a few specialized cases, such as the lampbrush and polytene chromosomes discussed above, most interphase chromosomes are too extended and entangled for their structures to be clearly seen. In contrast, the chromosomes from nearly all eucaryotic cells are readily visible during mitosis, when they coil up to form highly condensed structures. It is remarkable that this further condensation, which reduces the length of a typical interphase chromosome only about tenfold, produces such a dramatic change in the appearance of chromosomes.

Figure 4–52 depicts a typical **mitotic chromosome** at the metaphase stage of mitosis. The two daughter DNA molecules produced by DNA replication during interphase of the cell-division cycle are separately folded to produce two sister chromosomes, or *sister chromatids*, held together at their centromeres (see also Figure 4–21). These chromosomes are normally covered with a variety of molecules, including large amounts of RNA–protein complexes. Once this covering has been stripped away, each chromatid can be seen in electron micrographs to be organized into loops of chromatin emanating from a central scaffolding (Figures 4–53 and 4–54). Several types of experiment demonstrate that the order of visible features along a mitotic chromosome at least roughly reflects the order of the genes along the DNA molecule. Mitotic chromosome condensation can thus be thought of as the final level in the hierarchy of chromosome packaging (Figure 4–55).

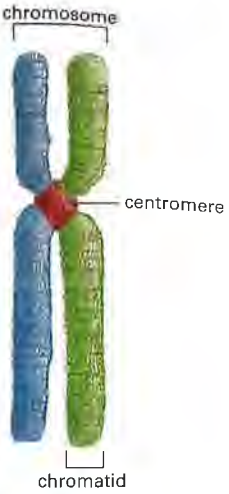


Figure 4–52 A typical mitotic chromosome at metaphase. Each sister chromatid contains one of two identical daughter DNA molecules generated earlier in the cell cycle by DNA replication.

Figure 4–53 A scanning electron micrograph of a region near one end of a typical mitotic chromosome. Each knoblike projection is believed to represent the tip of a separate looped domain. Note that the two identical paired chromatids drawn in Figure 4–52 can be clearly distinguished. (From M.P. Marsden and U.K. Laemmli, *Cell* 17:849–858, 1979. © Elsevier.)

The compaction of chromosomes during mitosis is a highly organized and dynamic process that serves at least two important purposes. First, when condensation is complete (in metaphase), sister chromatids have been disentangled from each other and lie side by side. Thus, the sister chromatids can easily separate when the mitotic apparatus begins pulling them apart. Second, the compaction of chromosomes protects the relatively fragile DNA molecules from being broken as they are pulled to separate daughter cells.

The condensation of interphase chromosomes into mitotic chromosomes occurs in M phase, and it is intimately connected with the progression of the cell cycle, as discussed detail in Chapters 17 and 18. It requires a class of proteins called *condensins* which using the energy of ATP hydrolysis, drive the coiling of each interphase chromosome that produces a mitotic chromosome. Condensins are large protein complexes that contain SMC proteins: long, dimeric protein molecules hinged in the center, with globular domains at each end that bind DNA and hydrolyze ATP (Figure 4–56). When added to purified DNA, condensins use the energy of ATP hydrolysis to make large right-handed loops in the DNA. Although it is not yet known how they act on chromatin, the coiling model shown in Figure 4–56C is based on the fact that condensins are a major structural component of mitotic chromosomes, with one molecule of condensin being present for every 10,000 nucleotides of mitotic DNA.

Figure 4–54 An electron micrograph of a mitotic chromosome. This chromosome (from an insect) was treated to reveal loops of chromatin fibers that emanate from a central scaffold of the chromatid. Such micrographs support the idea that the chromatin in all chromosomes is folded into a series of looped domains (see Figure 4–55). (Courtesy of Uli Laemmli.)

Each Mitotic Chromosome Contains a Characteristic Pattern of Very Large Domains

As mentioned earlier, the display of the 46 human chromosomes at mitosis is called the human karyotype. When stained with dyes such as Giemsa, mitotic

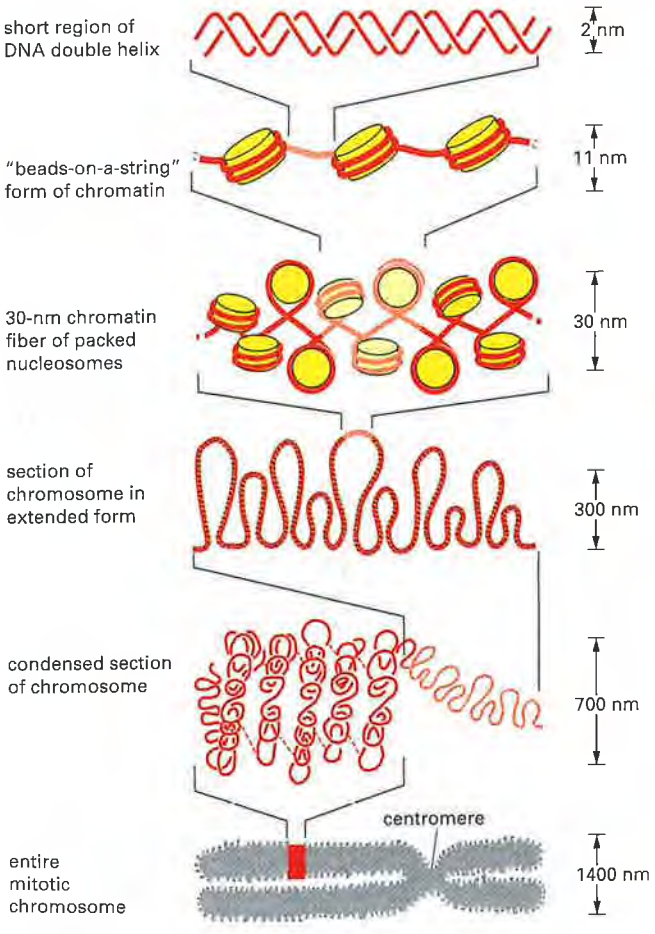


Figure 4–55 Chromatin packing. This model shows some of the many levels of chromatin packing postulated to give rise to the highly condensed mitotic chromosome.

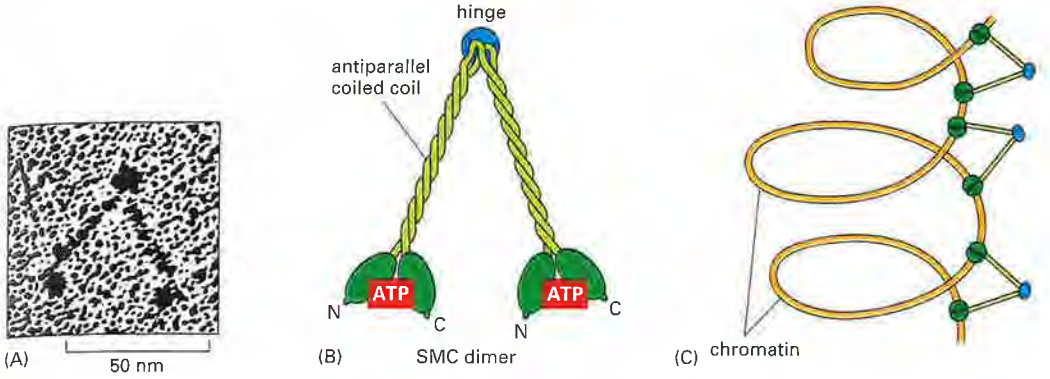


Figure 4–56 The SMC proteins in condensins. (A) Electron micrographs of a purified SMC dimer. (B) The structure of an SMC dimer. The long central region of this protein is an antiparallel coiled coil (see Figure 3–11) with a flexible hinge in its middle, as demonstrated by the electron micrograph in (A). (C) A model for the way in which the SMC proteins in condensins might compact chromatin. In reality, SMC proteins are components of a much larger condensin complex. It has been proposed that, in the cell, condensins coil long strings of looped chromatin domains (see Figure 4–55). In this way the condensins would form a structural framework that maintains the DNA in a highly organized state during M phase of the cell cycle. (A, courtesy of H.P. Erickson; B and C, adapted from T. Hirano, *Genes Dev.* 13:11–19, 1999.)

chromosomes show a striking and reproducible banding pattern along each chromosome, as shown in Figure 4–11. These bands are unrelated to those described earlier for the insect polytene chromosomes, which correspond to relatively small regions of interphase chromatin. In a human mitotic chromosome, all the chromatin is condensed and the bands represent a selective binding of the dyes.

By examining human chromosomes very early in mitosis, when they are less condensed than at metaphase, it has been possible to estimate that the total haploid genome contains about 2000 distinguishable bands. These coalesce progressively as condensation proceeds during mitosis, producing fewer and thicker bands. As we saw earlier, cytogeneticists routinely use the pattern of these chromosome bands to discover in patients genetic alterations such as chromosome inversions, translocations, and other types of chromosomal rearrangements (see Figure 4–12).

Mitotic chromosome bands are detected in chromosomes from species as diverse as humans and flies. Moreover, the pattern of bands in a chromosome has remained unchanged over long periods of evolutionary time. Each human chromosome, for example, has a clearly recognizable counterpart with a nearly identical banding pattern in the chromosomes of the chimpanzee, gorilla, and orangutan—although there are also clear differences, such as chromosome fusion, that give the human 46 chromosomes instead of the ape's 48 (Figure 4–57). This conservation suggests that chromosomes are organized into large domains that may be important for chromosomal function.

Even the thinnest of the bands in Figure 4–11 probably contains more than a million nucleotide pairs, which is nearly the size of a bacterial genome. These bands seem to reflect a rough division of chromosomes into regions of different GC content. The nucleotide sequence of the human genome has revealed large non-random blocks of sequence (some greater than 10^7 nucleotide pairs) that are significantly higher or lower in GC content than the genome-wide average of 41%. The blocks correlate roughly with the staining pattern of metaphase chromosomes. For example, bands that are darkly stained by Giemsa (the so-called G-bands) are correlated with DNA that is low in GC content, whereas lightly stained bands (the R-bands) correspond to DNA of higher than average GC content.

In general, GC-rich regions of the genome have a higher density of genes, especially of "house-keeping" genes, the genes that are expressed in virtually all cell types. On the basis of these observations, it has been proposed that the

Figure 4–57 Comparison of the Giemsa pattern of the largest human chromosome (chromosome 1) with that of chimpanzee, gorilla, and orangutan. Comparisons among the staining patterns of all the chromosomes indicate that human chromosomes are more closely related to those of chimpanzee than to those of gorilla and that they are more distantly related to those of orangutan. (Adapted from M.W. Strickberger, Evolution, 3rd edn. Sudbury, MA: Jones & Bartlett Publishers, 2000.)

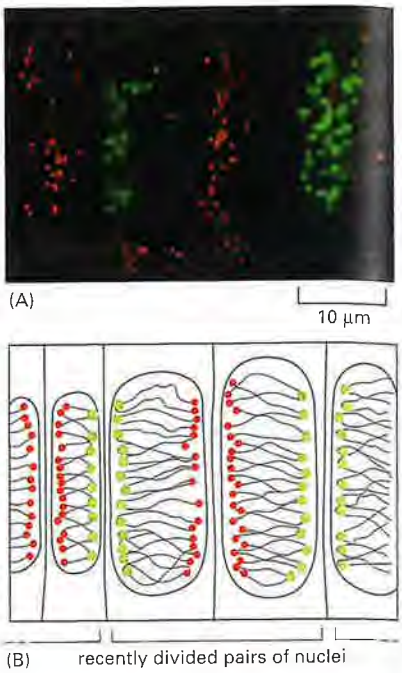


Figure 4–58 The polarized orientation of chromosomes found in certain interphase nuclei. (A) Fluorescent light micrograph of interphase nuclei from the rapidly growing root tip of a plant. Centromeres are stained *green* and teleomeres *red* by *in situ* hybridization of centromere- and telomere-specific DNA sequences coupled to the different fluorescent dyes. (B) Interpretation of (A) showing chromosomes in the Rabl orientation with all the centromeres facing one side of a nucleus and all the telomeres pointing toward the opposite side. (A, from R. Abranches et al., *J. Cell Biol.* 143:5–12, 1998. © The Rockefeller University Press.)

banding pattern may be related to gene expression. Perhaps the differentiation of chromosomes into G- and R-bands reflects subtle differences, determined by GC content, in the way in which chromatin loops are packaged in these areas. If this idea is correct, the rough division of chromosomes can be seen as a form of compartmentalization, in which the particular cellular components involved in gene expression are more concentrated in the R-bands where their activities are required. In any case, it should be obvious from this discussion that we are only beginning to glimpse the principles of large-scale chromosome organization.

Individual Chromosomes Occupy Discrete Territories in an Interphase Nucleus

We saw earlier in this chapter that chromosomes from eucaryotes are contained in the cell nucleus. However, the nucleus is not simply a bag of chromosomes; rather, the chromosomes—as well as the other components inside the nucleus which we shall encounter in subsequent chapters—are highly organized. The way in which chromosomes are organized in the nucleus during interphase, when they are active and difficult to see, has intrigued biologists since the nineteenth century. Although our understanding today is far from complete, we do know some interesting features of these chromosome arrangements.

A certain degree of chromosomal order results from the configuration that the chromosomes always have at the end of mitosis. Just before a cell divides, the condensed chromosomes are pulled to each spindle pole by microtubules attached to the centromeres; thus, as the chromosomes move, the centromeres lead the way and the distal arms (terminating in the telomeres) lag behind. The chromosomes in some nuclei tend to retain this so-called *Rabl orientation* throughout interphase, with their centromeres facing one pole of the nucleus and their telomeres pointing toward the opposite pole (Figures 4–58 and 4–59).

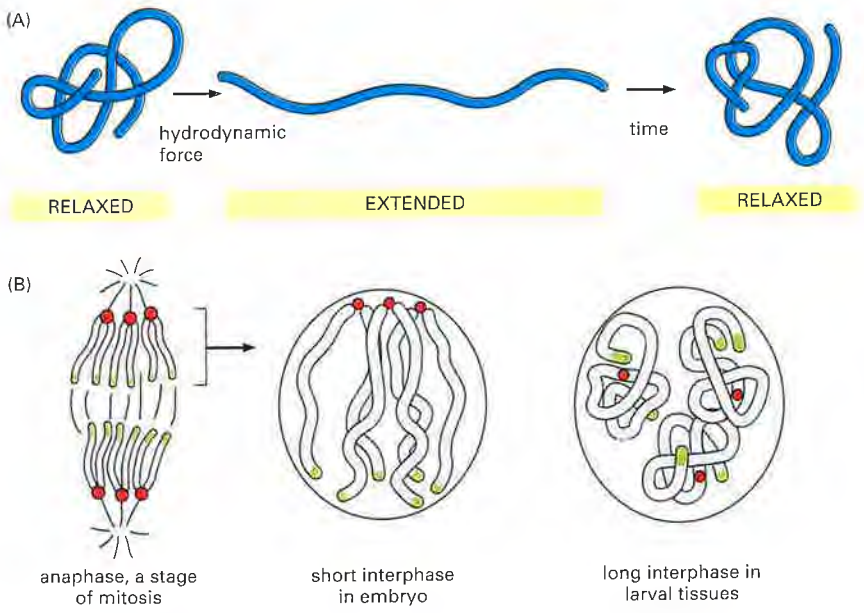


Figure 4–59 A polymer analogy for interphase chromosome organization. (A) The behavior of a polymer in solution. Entropy drives a long polymer into a compact conformation in the absence of an externally applied force. If the polymer is subjected to shear or hydrodynamic force, it becomes extended. But once the force is removed, the polymer chain returns to a more favorable, compact conformation. (B) The behavior of interphase chromosomes may reflect the same simple principles. In *Drosophila* embryos, for example, mitotic divisions occur at intervals of about 10 minutes; during the short intervening interphases, the chromosomes have little time to relax from the Rabl orientation induced by their movement during mitosis. However, in later stages of development, when interphase is much longer, the chromosomes have time to fold up. This folding may be strongly affected by specific associations between different regions of the same chromosome. (Adapted from A.F. Dernburg et al., *Cell* 85:745–759, 1996.)

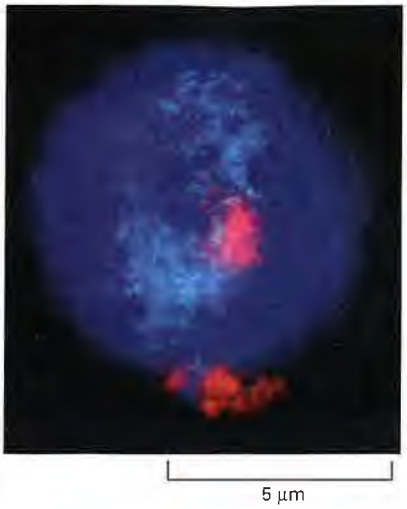


Figure 4–60 Selective "painting" of two interphase chromosomes in a human peripheral lymphocyte. The fluorescent light micrograph shows that the two copies of human chromosome 18 *(red)* and chromosome 19 *(turquoise)* occupy discrete territories of the nucleus. (From J.A. Croft et al., *J. Cell Biol.* 145:1119–1131, 1999. © The Rockefeller University Press.)

The chromosomes in most interphase cells are not found in the Rabl orientation; instead, the centromeres seem to be dispersed in the nucleus. Most cells have a longer interphase than the specialized cells illustrated above, and this presumably gives their chromosomes time to assume a different conformation (see Figure 4–59). Nevertheless, each interphase chromosome does tend to occupy a discrete and relatively small territory in the nucleus: that is, the different chromosomes are not extensively intertwined (Figure 4–60).

One device for organizing chromosomes in the nucleus may be the attachment of certain portions of each chromosome to the nuclear envelope (Figure 4–61). For example, in many cells, telomeres seem bound in this way. But the exact position of a chromosome in a nucleus is not fixed. In the same tissue, for example, two apparently identical cells can have different chromosomes as nearest neighbors.

Some cell biologists believe that there is an intranuclear framework, analogous to the cytoskeleton, on which chromosomes and other components of the nucleus are organized. The *nuclear matrix*, or *scaffold*, has been defined as the insoluble material left in the nucleus after a series of biochemical extraction steps. Some of the proteins that constitute it can be shown to bind specific DNA sequences called *SARs* or *MARs* (scaffold-associated or matrix-associated regions). These DNA sequences have been postulated to form the base of chromosomal loops (see Figure 4–44), or to attach chromosomes to the nuclear envelope and other structures in the nucleus. By means of such chromosomal attachment sites, the matrix might help to organize chromosomes, localize genes, and regulate gene expression and DNA replication. It still remains uncertain, however, whether the matrix isolated by cell biologists represents a structure that is present in intact cells.

Summary

Chromosomes are generally decondensed during interphase, so that their structure is difficult to visualize directly. Notable exceptions are the specialized lampbrush chromosomes of vertebrate oocytes and the polytene chromosomes in the giant secretory cells of insects. Studies of these two types of interphase chromosomes suggest that each long DNA molecule in a chromosome is divided into a large number of discrete domains organized as loops of chromatin, each loop probably consisting of a folded 30-nm chromatin fiber. When genes contained in a loop are expressed, the loop decondenses and allows the cell's machinery easy access to the DNA.

Euchromatin makes up most of interphase chromosomes and probably corresponds to looped domains of 30-nm fibers. However, euchromatin is interrupted by stretches of heterochromatin, in which 30-nm fibers are subjected to additional levels of packing that usually render it resistant to gene expression. Heterochromatin is commonly found around centromeres and near telomeres, but it is also present at other positions on chromosomes. Although considerably less condensed than mitotic chromosomes, interphase chromosomes occupy discrete territories in the cell nucleus; that is, they are not extensively intertwined.

All chromosomes adopt a highly condensed conformation during mitosis. When they are specially stained, these mitotic chromosomes have a banding structure that allows each individual chromosome to be recognized unambiguously. These bands contain millions of DNA nucleotide pairs, and they reflect a poorly-understood coarse heterogeneity of chromosome structure.

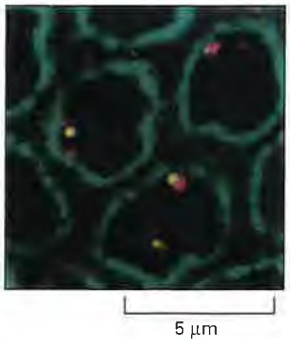


Figure 4–61 Specific regions of interphase chromosomes in close proximity to the nuclear envelope. This high-resolution microscopic view of nuclei from a *Drosophila* embryo shows the localization of two different regions of chromosome 2 *(yellow* and *magenta)* close to the nuclear envelope (stained green with antilamina antibodies). Other regions of the same chromosome are more distant from the envelope. (From W.F. Marshall et al., *Mol. Biol. Cell* 7:825–842, 1996.)

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EXHIBIT 5

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Frontiers in Research Review: Cutting-Edge Molecular Approaches to Therapeutics

ANTISENSE OLIGONUCLEOTIDES: FROM DESIGN TO THERAPEUTIC APPLICATION

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SUMMARY

1. An antisense oligonucleotide (ASO) is a short strand of deoxyribonucleotide analogue that hybridizes with the complementary mRNA in a sequence-specific manner via Watson–Crick base pairing. Formation of the ASO–mRNA heteroduplex either triggers RNase H activity, leading to mRNA degradation, induces translational arrest by steric hindrance of ribosomal activity, interferes with mRNA maturation by inhibiting splicing or destabilizes pre-mRNA in the nucleus, resulting in down-regulation of target protein expression.

2. The ASO is not only a useful experimental tool in protein target identification and validation, but also a highly selective therapeutic strategy for diseases with dysregulated protein expression.

3. In the present review, we discuss various theoretical approaches to rational design of ASO, chemical modifications of ASO, ASO delivery systems and ASO-related toxicology. Finally, we survey ASO drugs in various current clinical studies.

Key words: antisense oligonucleotide design, cell-penetrating peptide, dendrimer, gapmer antisense oligonucleotide, liposome, locked nucleic acid, peptide nucleic acid, phosphoroamidate morpholino oligomer, phosphorothioate, RNase H.

INTRODUCTION

An antisense oligonucleotide (ASO) is a single-stranded deoxyribonucleotide (typically 20 bp in length) that is complementary to the target mRNA. Hybridization of ASO to the target mRNA via Watson–Crick base pairing can result in specific inhibition of gene expression by various mechanisms, depending on the chemical make-up of the ASO and location of hybridization, resulting in reduced levels of translation of the target transcript.[1] The ASO is not only a useful tool for studies of loss-of-gene function and target validation, but also highly valuable as a novel therapeutic strategy to treat any disease that is linked to dysregulated gene expression. Antisense oligonucleotide-induced protein knockdown is usually achieved by induction of RNase H endonuclease activity that cleaves the RNA–DNA heteroduplex. This leads to the degradation of target mRNA while leaving the ASO intact.[2] Other ASO mechanisms include translational arrest by steric hindrance of ribosomal activity, interference with mRNA maturation by inhibiting splicing and destabilization of pre-mRNA in the nucleus[3] (Fig. 1).

In the present review, we first discuss a few computational algorithms in ASO design. There are other screening strategies to obtain potent ASO, such as mRNA walking,[4] oligonucleotide array[5] and RNase H mapping,[6] but these approaches are more costly and labour intensive and require expensive automation equipment that many small laboratories may not be able to afford. Rational design of ASO based on computational algorithms that are freely available in the public domain is the most economical approach to ASO design and very often generates potent ASO from a handful of candidates. Because there is no stand-alone program in predicting highly potent ASO, one can increase the 'hit rate' using several computer software packages.' Unmodified ASOs are susceptible to degradation by nucleases. Therefore, different chemical modifications of ASO have been developed to decrease nuclease cleavage and increase the biostability and potency of the ASO. We then discuss the latest approaches for ASO delivery *in vitro* and *in vivo*. Owing to inherent ionic charges of ASO, it is difficult for the ASO to cross the plasma membrane efficiently. As such, the ASO needs to be coupled to a carrier for efficient membrane binding and internalization. Finally, toxicology and clinical studies of ASOs are discussed.

ANTISENSE OLIGONUCLEOTIDE DESIGN

The strength and stability of interactions between the ASO and complementary target mRNA depends on factors such as thermodynamic stability, the secondary structure of the target mRNA transcript and the proximity of the hybridization site to functional motifs on the designated transcript, such as the 5′ CAP region or translational start site. We need to consider at least four parameters in ASO design in order to increase the 'hit rate': (i) prediction of the secondary structure of the RNA; (ii) identification of preferable RNA secondary local structures; (iii) motifs searching and GC content calculation; and (iv) binding energy (ΔG°_{37}) prediction.

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List of abbreviations:

ASO	Antisense oligonucleotide	2′-OMe	2′-*O*-Methyl
DOPE	Dioleyphosphatidylethanolamine	PMO	Phosphoroamidate morpholino oligomer
LNA	Locked nucleic acid	PNA	Peptide nucleic acid
2′-MOE	2′-*O*-Methoxyethyl	PS	Phosphorothioate

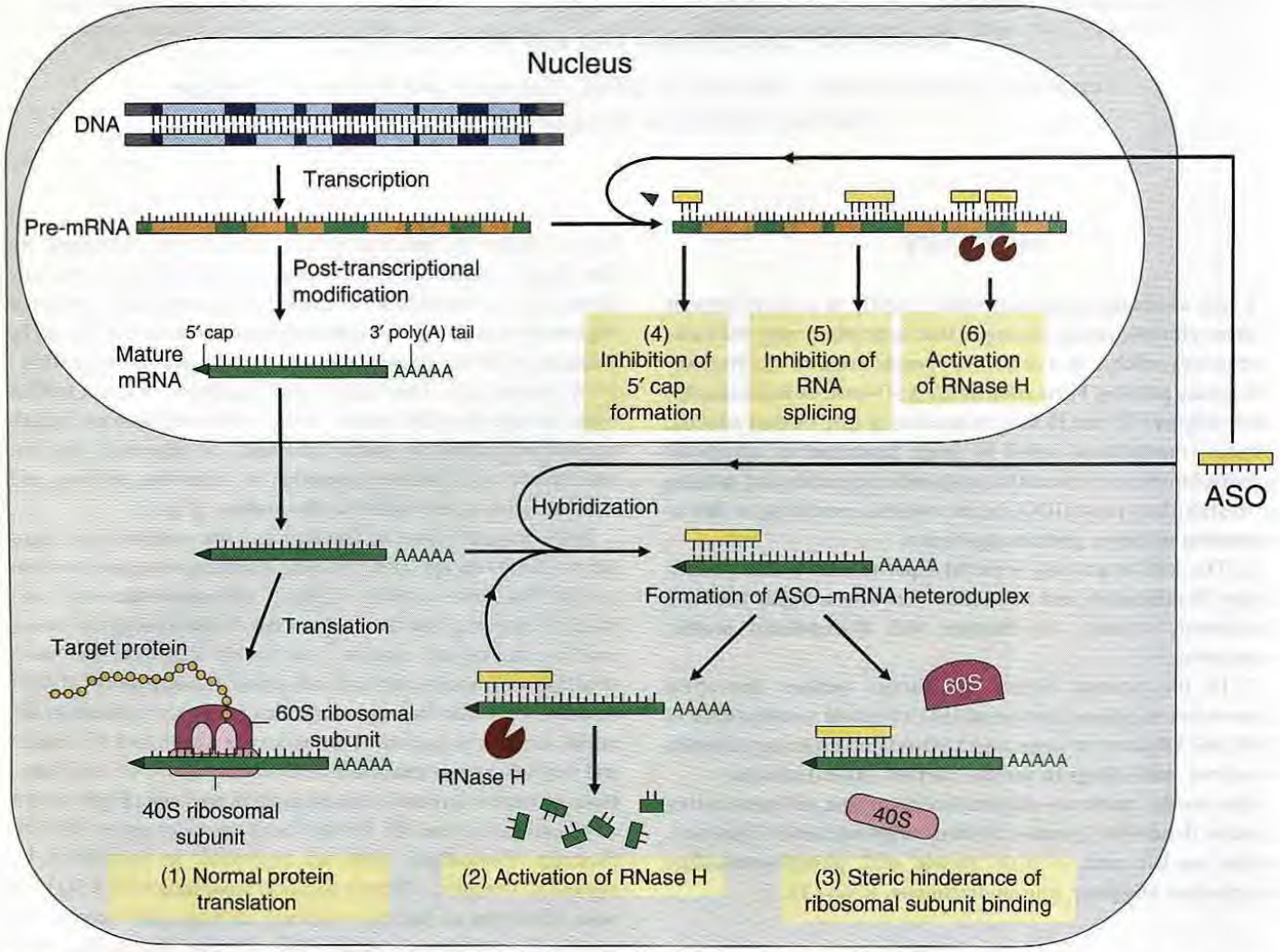

Fig. 1 Modes of action of antisense oligonucleotides (ASOs). In the absence of ASO, normal gene and protein expression is maintained. The ASO is taken up by cellular endocytosis and can hybridize with target mRNA in the cytoplasm. Formation of an ASO–mRNA heteroduplex induces (2) activation of RNase H, leading to selective degradation of bound mRNA or (3) steric interference of ribosomal assembly. Both actions will result in target protein knockdown. Alternatively, the ASO can enter the nucleus and regulate mRNA maturation by (4) inhibition of 5′ cap formation, (5) inhibition of mRNA splicing and (6) activation of RNase H. Theoretically, the ASO can selectively knock down any target gene and protein expression, leading to therapeutic benefit.

Prediction of the secondary structure of RNA

It is generally accepted that effective ASO design depends on accurate prediction of the secondary structure of the RNA.[7,8] However, a truly reliable algorithm to predict any single mRNA secondary structure and folding pattern is lacking. A widely used *m*fold program is available in the public domain (http://www.bioinfo.rpi.edu/applications/mfold) that predicts all possible optimal and suboptimal structures of a particular sequence of mRNA. The core algorithm predicts overall minimum free energy, ΔG, of different possible folding.[9] Conversely, another commonly used computer algorithm is the *s*fold program (http://sfold.wardsworth.org/index.pl), which predicts only the best secondary structure of the target transcript.[10] Using a combination of both *m*fold and *s*fold, one can determine the most frequently occurring secondary structure of the target mRNA with minimal overall free energy as a potential ASO target site.

Identification of preferable mRNA local secondary structures

An effective ASO should be designed at the regions where mRNA is accessible for hybridization.[6,11] Local structures accessible to ASOs are those usually located at the terminal end, internal loops, joint sequences, hairpins and bulges of 10 or more consecutive

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nucleotides.[12] In conjunction with *m*fold, a new software (TargetFinder; http://www.bioit.org.cn/ao/targetfinder.com) has recently been developed to facilitate ASO target site selection based on the method of mRNA accessible site tagging (MAST).[13] Yang *et al.*[14] have recently demonstrated that potent ASO target sites can be found in highly conserved local motifs, whereas ASO targeting at variable local motifs may lead to non-sequence specific effects. Therefore, in order to increase the 'hit rate' of potent ASO design, one needs to look for locally conserved structures among various optimal and suboptimal mRNA predicted secondary structures.

Motifs determination and GC content calculation

After confirmation of the accessible conserved local secondary structures and the corresponding sequences of ASOs (approximately 20 bp), one can settle on some well-defined activity enhancing motifs and discard those activity decreasing motifs in the ASOs.[15] Matveeva *et al.*[15] analysed data collected from > 1000 experiments using phosphorothioate (PS)-modified ASOs and found a positive correlation between ASO-mediated mRNA knockdown and the presence of CCAC, TCCC, ACTC, GCCA and CTCT motifs in the ASOs. Conversely, the presence of GGGG (G-quartets formation), ACTG, AAA and TAA motifs in ASOs weakened ASO activity. Although it is believed that the formation of the ASO–mRNA heteroduplex stimulates RNase H activity, leading to target mRNA degradation, Ho *et al.*[16] found that RNase H activity is sequence independent. Instead, GC content is strongly correlated with the thermodynamic stability of the ASO–mRNA duplexes and RNase H activity. Ho *et al.*[16] observed strong ASO effects with a minimum of 11 G or C residues in 20 bp ASOs, whereas poor inhibition was observed by ASOs having nine or fewer G or C residues.

Binding energy (ΔG°_{37}) prediction

Successful ASO design also needs to take into consideration thermodynamic energy. Software for calculating thermodynamic properties between the ASO and mRNA target sequence is available.[17] The program OligoWalk from the package RNAstructure 3.5 (http://128.151.176.70/RNAstructure.html) has been developed to calculate binding energy of ASO/ASO and ASO/mRNA. To design a potent ASO, the binding energy between the ASO and mRNA should be $\Delta G^\circ_{37} \geq -8$ kcal/mol, whereas the energy for binding between ASOs should be $\Delta G^\circ_{37} \geq -1.1$ kcal/mol.[15] By using two large databases from ISIS Pharmaceuticals (Carlsbad, CA, USA) and the published literature, Matveeve *et al.*[18] showed that the hit rate of developing a potent and active ASO is six- and threefold higher, respectively, if the above criteria are met. In addition, by using this algorithm alone, Fei and Zhang[19] were able to design ASO for the downregulation of vascular endothelial growth factor protein expression with a success rate higher than 85%.

To circumvent a tedious ASO design approach using multiple computer algorithms, a fast and handy ASO prediction based on a neural network has been developed using on a broad range of parameters, including base composition, RNA–ASO binding energy, RNA–ASO terminal properties, ASO–ASO binding properties and 10 verified sequence motifs correlated with efficacy and Rnase H accessibilities.[20] The prediction server interface is available at http://www.cgb.ki.se/AOpredict. Although this model can predict effective ASOs with > 50% gene expression inhibition with a success rate of 92%, some effective sequences may be missed because the selective criteria of this program are too stringent. Furthermore, there is a lack of thermodynamic consideration in this network in correlating dimer energy with efficacy.

In conclusion, there is no reliable stand-alone algorithm to accurately predict ASO. In practice, ASOs have to be tried and screened, so that some companies, such as ISIS Pharmaceuticals, have performed gene-walking and have screened hundreds of ASOs against one gene. Using this linear 'shot-gun' approach, only 2–5% of the oligonucleotides are generally found to be potent ASOs.[4] However, by combining the above theoretical criteria using multiple computational algorithms, one can markedly increase the hit rate of highly potent ASOs.

CHEMICAL MODIFICATIONS OF ASO

An unmodified ASO is rapidly attacked by all types of nucleases in biological fluid and its overall charged property prevents it from penetrating through the cell membrane. Various chemical modifications have been developed to enhance nuclease resistance, prolong tissue half-life, increase affinity and potency and reduce non-sequence-specific toxicity (Fig. 2).

First-generation ASOs

First generation ASOs are those containing a PS-modified backbone, in which one of the non-bridging oxygen atoms in the phosphodiester bond is replaced by a sulphur atom.[21] Phosphorothioate modification confers higher resistance to the ASO against nuclease degradation, leading to higher bioavailability of the oligonucleotide. Phosphorothioate-modified ASOs promote RNase H-mediated cleavage of target mRNA. However, this modification may slightly reduce the affinity of the ASO for its mRNA target because the melting temperature of the ASO–mRNA heteroduplex decreases by approximately 0.5°C per nucleotide.[22] Phosphorothioate-modified ASOs have also been reported to produce non-specific effects by interactions with cell surface and intracellular proteins.[3] Despite these disadvantages, PS modification is the most widely performed chemical modification of ASOs for loss-of-function studies *in vitro* and *in vivo* for gene target identification and validation. Indeed, intravitreous fomivirsen, a 21 bp first generation PS-modified ASO, is currently the only ASO drug approved for clinical use.[23]

Second-generation ASOs

To further enhance nuclease resistance and increase binding affinity for target mRNA, second-generation ASOs with 2′-alkyl modifications of the ribose were developed. 2′-*O*-Methyl (2′-OMe) and 2′-*O*-Methoxyethyl (2′-MOE) modifications of PS-modified ASOs are the two most widely studied second-generation ASOs.[1] Unexpectedly, 2′-OMe and 2′-MOE substitutions do not support RNase H-mediated cleavage of target mRNA, which dampens the efficacy of the ASO.[24] To circumvent this shortcoming, a chimeric ASO was developed in which a central 'gap' region consisting of approximately 10 PS-modified 2′-deoxynucleotides is flanked on both sides (5′ and 3′ directions) by approximately five nucleotide 'wings'. The wings are composed of 2′-OMe or 2′-MOE PS-modified nucleotides.[25] This chimeric 'gapmer' ASO allows RNase H to sit in the central gap to execute target-specific mRNA degradation; meanwhile, the 'wings' resist nuclease cleavage of ASO by 2′-alkyl modifications at both

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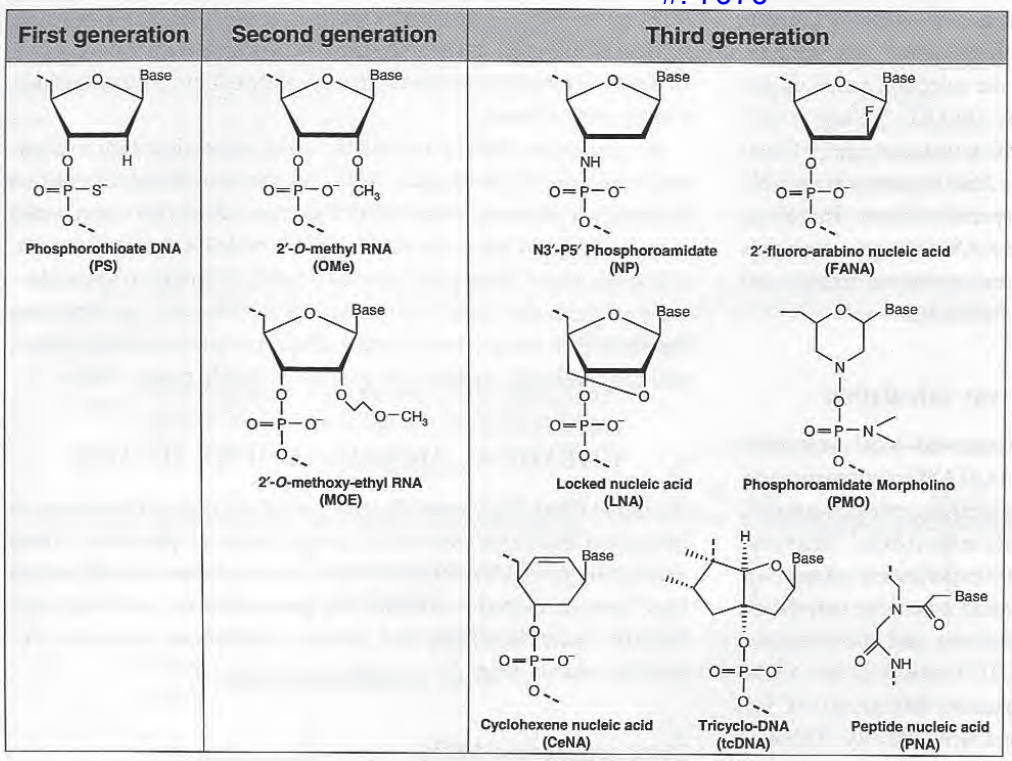

Fig. 2 Chemical modifications of antisense oligonucleotides.

ends. Extensive studies have been performed *in vivo* to assess the stability and toxicity of these modified ASOs.[1,26]

Third-generation ASOs

To further enhance target affinity, nuclease resistance, biostability and pharmacokinetics, a third generation of ASO was developed mainly by chemical modifications of the furanose ring of the nucleotide. Peptide nucleic acid (PNA), locked nucleic acid (LNA) and phosphoroamidate morpholino oligomer (PMO) are the three most studied third-generation ASOs.[3,27]

Peptide nucleic acid is a synthetic DNA mimic in which the phosphodiester backbone is replaced with a flexible pseudopeptide polymer (*N*-(2-aminoethyl)glycine) and nucleobases are attached to the backbone via methylene carbonyl linkage.[28,29] Peptide nucleic acid is a non-charged nucleotide analogue that can hybridize complementary DNA or RNA with higher affinity and specificity than unmodified DNA–DNA and DNA–RNA duplexes. In addition, PNA demonstrates high biostability in biological fluid because it is not degraded by nucleases or peptidases. Peptide nucleic acid exerts its antisense effect by forming a sequence-specific duplex with mRNA, which mainly causes steric hindrance of translational machinery leading to protein knockdown because it is not a substrate for RNase H. Furthermore, PNA can elicit antigene effects by hybridizing with double-stranded DNA in four possible configurations, including triplex, triplex invasion, duplex invasion and double duplex invasion,[29,30] resulting in transcriptional arrest. Substantial data have revealed the effectiveness of PNA in gene silencing in various *ex vivo* models and in genetic and cytogenetic analyses,[30–32] but its efficacy *in vivo* remains to be determined.

Locked nucleic acid is a conformationally restricted nucleotide containing a 2′-*O*,4′-*C*-methylene bridge in the β-D-ribofuranosyl configuration. This modification greatly enhances its hybridization affinity towards target mRNA and DNA, with a substantial increase in the thermal stability of the duplexes.[33] In addition, LNA is resistant to nuclease degradation. Like any 2′-*O* ribose modification, LNA is not a substrate for RNase H. Notwithstanding, LNA monomer can be freely incorporated into RNA and DNA to form chimeric oligonucleotides resulting in restoration of RNase H-mediated cleavage of mRNA. It has been shown that the chimeric LNA/DNA/LNA gapmer with seven to 10 PS-modified DNA central gaps flanked by three to four LNA oligomers on both ends provides highly efficient mRNA cleavage, in addition to high ASO potency, target accessibility and nuclease resistance.[34] Among the nine members of the LNA molecular family, α-L-LNA is the stereoisomer of β-D-LNA and has been shown to demonstrate the highest efficacy in mRNA knockdown in both *in vitro* and *in vivo* studies, making it one of the most promising LNA antisense agents.[35–37]

Phosphoroamidate morpholino oligomer represents a non-charged ASO agent in which the ribose sugar is replaced by a six-membered morpholino ring and the phosphodiester bond is replaced by a phosphoroamidate linkage.[38] Phosphoroamidate morpholino oligomer does not support RNaes H activity, such that its ASO effect is primarily mediated by steric interference of ribosomal assembly resulting in translational arrest. This chemical modification also confers excellent resistance to nucleases and proteases in biological fluid. Phosphoroamidate morpholino oligomer does not readily enter mammalian cells in culture, but a recent study using an arginine-rich peptide (ARP) conjugation to PMO markedly enhanced its cellular uptake and antisense potency by increasing the thermal stability of the ARP–PMO–mRNA heteroduplex.[39] Phosphoroamidate morpholino oligomer has demonstrated antisense efficacy in animal models *in vivo* and in human clinical trials.[40,41]

DELIVERY OF ASO

Unmodified naked ASO has a net negative charge and can barely penetrate the plasma membrane. Cellular uptake of ASO is primarily

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an adsorptive endocytosis process.[42] Phosphorothioate modification of ASOs not only enhances nuclease resistance, but also promotes adsorption of ASOs to cell surface proteins, resulting in higher internalization of the ASOs. Peptide nucleic acid and PMO are non-charged oligonucleotides that do not interact well with cell surface proteins, making them even more difficult for adsorptive endocytosis. The amount of ASO that enters cells is so low that a variety of delivery strategies has been devised to enhance cellular uptake of ASOs and the ensuing mRNA knockdown (for a review, see Lysik and Wu-Pong[42]). Mechanical techniques, like electroporation and micro-injection, are very useful in delivering ASOs into cell cultures *in vitro*, but are impractical for *in vivo* studies. In contrast, chemical-mediated ASO delivery has been tested extensively in both *in vitro* and *in vivo* studies. Cationic lipid carriers like *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulphate (DOTAP) are the most widely used vectors for ASO internalization. Upon entry into the intracellular milieu in the form of an endosome, the ASO needs to escape from the endosomal vesicles to avoid lysosomal degradation so as to interact directly with target mRNA in the cytosol and in the nucleus. Dioleylphosphatidylethanolamine (DOPE) has been added into the lipid carrier formulation to promote endosomal membrane destabilization, leading to release of the ASO into the cytosol.[42,43] Indeed, a pH-sensitive fusogenic liposome preparation consisting of DOPE and an amphipathic lipid, such as cholesteyl hemisuccinate, supports fusion of the liposomal and endosomal membranes at a pH below 5.5, resulting in ASO escape and enhanced mRNA knockdown efficacy.[43] The use of microparticles, such as biodegradable copolymer poly(D,L-lactide-coglycolide) in sustained-release ASO delivery has also been investigated. The ASOs, encapsulated in microspheres ranging from 10 to 60 μm, are released gradually with enhanced serum stability, increased cytosolic and nuclear delivery and prolonged duration of ASO action in both *in vitro* and *in vivo* models.[42,44]

Covalent conjugation of ASO to a macromolecule like dendrimer[42,45] and to cell-penetrating peptides (CPP)[46] has been shown to promote cellular uptake of the ASO. Dendrimers are spherical and highly branched polymers with cationic polyamidoamine moieties capable of forming a covalent complex with the ASO. In contrast with the liposome formulation, the dendrimer–ASO complex is stable and active in the presence of serum. It enhances ASO cellular delivery into the cytosol and nucleus and increases the retention time of ASO in the cells. Conversely, CPP is a short peptide sequence (< 30 amino acids) with net positive charge that allows rapid translocation of a large molecule like the ASO through the cell membrane via an energy dependent pathway. Commonly used CPP include penetratin (RQIKIWFQNRRMKWKK), HIV TAT peptide 48–60 (GRKKRRQRRRPPQ) and transportan (GWTLNSAGYLLGKINLKALAALAKKIL-amide). The ASOs can be directly conjugated to any of these CPP via formation of disulphide bridge. By far, PNA is the most frequently used ASO in evaluating CPP-mediated mRNA knockdown in both *in vitro* and *in vivo* studies.[39,46,47]

Despite the advances in liposome technology, the most critical challenge for the ASO to be an effective therapeutic is for it to be delivered to the site of action and to produce expected efficacy *in vivo*. There is a new trend of using topical application of ASOs as the most popular mode of administration. In fact, the first clinically approved ASO, formivirsen, is administered intravitreously. More recent studies have revealed that alicaforsen, a PS-modified inter-cellular adhesion molecule-1 ASO, produced promising acute and long-term benefits in ulcerative colitis patients when given locally in an enema preparation,[48] AP 12009, a PS-modified transforming growth factor-β2 ASO, induced prolonged and complete brain tumour remission when given directly into the brain[49] and TPI-ASM8, an ASO targeted to cytokine receptors, is being evaluated as an inhalational therapy for asthma.

TOXICOLOGY OF ASO

All three generations of ASOs have gone through preclinical toxicological studies and, in fact, some of these ASOs have entered clinical trials.[1,3,27] In general, ASO drugs produce dose-dependent, transient and mild-to-moderate toxicities manifested in rodents, primates and humans. The most common acute toxicities associated with ASO administration *in vivo* are activation of the transient complement cascade and inhibition of the clotting cascade. Both these toxic effects are dependent on ASO backbone chemistry, but are ASO sequence independent. The toxicities are largely due to the non-specific binding properties of PS-ASOs to proteins at high plasma concentrations. The PS-ASO may interact with factor H, a circulating negative regulatory factor, thus facilitating activation of the complement cascade via an alternative pathway, resulting in increased complement split products, such as C3a and C5a, and subsequent cardiovascular events, such as hypotension. Conversely, PS-ASO, with its polyanionic characteristics, binds to multiple coagulation factors, such as VIIIa, IXa X and II, leading to a transient self-limited prolongation of activated partial thromboplastin times.[1,50,51] Another frequently occurring subchronic toxicity is immune stimulation, manifested as splenomegaly, lymphoid hyperplasia and diffused multi-organ mixed mononuclear cell infiltrates.[50] This is due to an unmethylated cytosine–phosphorous–guanine (CpG) motif in the ASO sequence that can be recognized by Toll-like receptor-9 in immune cells, resulting in the release of cytokines (interleukin (IL)-6, IL-12 and interferon-γ), B cell proliferation, antibody production and activation of T lymphocyte and natural killer (NK) cells.[52] The immunostimulatory effects of CpG are further amplified when the ummethylated CpG is flanked by two 5′ purines and two 3′ pyrimidines (e.g. AACGTT).[50] Newer generations of ASOs have been designed to circumvent this side-effect by exclusion of the CpG motif or by methylation of cytosine to reduce the immune stimulatory effects. In addition, introduction of LNA into the PS-ASO has been shown to reduce, and even eliminate, CpG dinucleotide-mediated immune stimulation.[53] Other mild and self-limiting toxicities usually observed at high plasma ASO concentrations are thrombocytopenia, enhanced liver enzyme (e.g. aspartate aminotransferase and alanine aminotransferase) levels and hyperglycaemia.[51]

ANTISENSE OLIGONUCLEOTIDE DRUGS IN CLINICAL TRIALS

At present, formivirsen (Vitravene; Isis Pharmaceuticals) is the first and only US Food and Drug Administration (FDA)-approved ASO drug indicated for the treatment of peripheral cytomegalovirus retinitis in AIDS patients by local intravitreous administration.[23] With the approval of this novel therapeutic strategy, numerous clinical trials (mainly on first-generation ASO drugs) have been conducted for the treatment of diseases such as cancer, viral infections, autoimmune disorders and allergic asthma[1,3,27] (Table 1). Although most of the

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Table 1 Antisense oligonucleotides in clinical trials

ASO	Gene target	Generation	Delivery
LErafAON-ETU	*c-raf-1*	PS	Systemic
Oblimerson (G3139)	*Bcl-2*	PS	Systemic
RESTEN-MP	*c-myc*	PMO	Systemic
AVI-4126	*c-myc*	PMO	Systemic
OGX-011	Clusterin	2′-MOE	Systemic
GTI-2040	Ribonucleotide reductase	PS	Systemic
TPI-ASM8	Cytokine receptors	PS	Aerosol
ASO	Dystrophin	PMO	Systemic
AP 12009	TGF-β2	PS	Intratumoral
Alicaforsen	ICAM-1	PS	Systemic/enema
ISIS 104838	TNF-α	2′-MOE	Subcutaneous
ISIS 3521	PKCα	PS	Systemic

ASO, antisense oligonucleotide; PS, phosphorothioate; PMO, phosphoroamidate morpholino oligomer; 2′-MOE, 2′-*O*-methoxyethyl; TGF-β2, transforming growth factor-β2; ICAM-1, intercellular adhesion molecule-1; TNF-α, tumour necrosis factor-α; PKCα, protein kinase Cα.

first-generation ASOs failed to survive the clinical trials, partly due to suboptimal pharmacokinetic, pharmacodynamic and toxicological profiles, there are a few first-generation ASOs that have demonstrated promising therapeutic potential. One of these is an 18 bp PS-ASO oblimersen (G3139; Genasense; Genta Incorporated, Berkeley Heights, NJ, USA) targeting the first six codons of the *bcl-2* mRNA open reading frame, which has shown promising therapeutic effects in various types of cancer. *Bcl-2* is overexpressed in cancer cells, controls the anti-apoptotic pathway and contributes tumour resistance to chemotherapy. By knocking down bcl-2 protein, oblimersen increases the efficacy of cytotoxic agents in the treatment of cancers such as multiple myeloma and chronic lymphocytic leukaemia.[54,55] Another PS-ASO, namely GTI-2040, is a 20 bp PS-ASO targeting the coding region of human ribonucleotide reductase (RNR) R2 subunit component mRNA. The RNR is composed of R1 and R2 components and is critical for the production of 2′-deoxyribonucleoside 5′-triphosphates required for DNA synthesis.[56] The R2 subunit appears to determine the malignant potential of tumour cells via positive cooperation with activated oncogenes such as *c-myc* and *H-ras*. Overexpression of the R2 subunit is also associated with an increased drug resistance property of cancer cells. It has been shown that GTI-2040 inhibits the growth of various tumours in xenograft models.[56] Phase I/II trials using GTI-204 in combination with anticancer drugs in patients with various types of cancer are currently in progress.[57] Another first-generation ASO currently in clinical trial is LErafAON (NeoPharm, Waukegan, IL, USA), a cationic liposome-encapsulated *raf-1* proto-oncogene ASO. Dsyregulated raf-1 activity has been implicated in oncogenic transformation, increased resistance to cytotoxic agents and angiogenesis in human tumours. The ASO targeted at raf-1 inhibits tumour growth and increases sensitivity to both chemotherapy and radiotherapy. A phase I study[58] of LErafAON revealed a hypersensitivity reaction and dose-dependent thrombocytopenia. Future clinical trials of this ASO depend on an improvement of the liposomal formulation.[58]

In addition, newer generations of ASO have entered into clinical trials. OGX-011 is a second-generation 2′-MOE gapmer ASO targeting the translation initiation site of human clusterin mRNA. Clusterin plays a critical role in tumorigenesis and disease progression. It is upregulated in various types of cancer, such as prostate cancer and breast cancer, and in response to stress, such as radiation therapy and chemotherapy.[59,60] Clusterin overexpression confers resistance to anticancer treatments, whereas ASO-mediated clusterin knockdown enhanced the efficacy of anticancer agents. A phase I study of OGX-011 in patients with localized prostate cancer showed that it was well-tolerated and reduced clusterin expression in primary prostate tumours.[59] AVI-4126 is a non-charged 20 bp third-generation PMO targeting *c-myc* mRNA. The c-myc protein is upregulated in human solid tumours, such as prostate cancer and breast cancer, leukaemias and lymphomas, and plays a critical role in controlling cell proliferation, differentiation and apoptosis. AVI-4126 has demonstrated beneficial effects in tumour models of lung cancer and prostate cancer.[41,61] A phase I clinical study of AVI-4126 revealed significant tumour accumulation of PMO in breast and prostate tissues and a favourable safety profile devoid of common side-effects,[41] such as activation of the complement cascade, inhibition of the clotting cascade, thrombocytopenia and hypotension associated with PS-ASO.[41,61]

With a better understanding of ASO design, chemical modifications of ASO, ASO delivery systems and human pharmacokinetic and pharmacodynamic profiles of ASO and numerous ASO candidates being tested in both preclinical and clinical stages for a variety of human diseases,[1,3,27] the prospect for ASOs to become a major therapeutic modality is very bright.

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Review

Gene Knockdowns in Adult Animals: PPMOs and Vivo-Morpholinos

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Abstract: Antisense molecules do not readily cross cell membranes. This has limited the use of antisense to systems where techniques have been worked out to introduce the molecules into cells, such as embryos and cell cultures. Uncharged antisense bearing a group of guanidinium moieties on either a linear peptide or dendrimer scaffold can enter cells by endocytosis and subsequently escape from endosomes into the cytosol/nuclear compartment of cells. These technologies allow systemic administration of antisense, making gene knockdowns and splice modification feasible in adult animals; this review presents examples of such animal studies. Techniques developed with PPMOs, which are an arginine-rich cell-penetrating peptide linked to a Morpholino oligo, can also be performed using commercially available Vivo-Morpholinos, which are eight guanidinium groups on a dendrimeric scaffold linked to a Morpholino oligo. Antisense-based techniques such as blocking translation, modifying pre-mRNA splicing, inhibiting miRNA maturation and inhibiting viral replication can be conveniently applied in adult animals by injecting PPMOs or Vivo-Morpholinos.

Keywords: Antisense; Morpholino; PPMO; Vivo-Morpholino.

1. Introduction

Morpholino oligos are steric-blocking antisense molecules which bind to RNA and get in the way of cellular processes. These oligos have no electrical charge, do not interact strongly with proteins, and do not require the activity of RNase-H, Argonaute, or other catalytic proteins for their activity.

Antisense molecules however do not readily cross cell membranes without delivery techniques, which has prevented their effective use in adult animals [1]. With delivery of Morpholino oligos achieved *in vivo* by Vivo-Morpholinos and Morpholino oligos linked to arginine-rich cell penetrating peptides (PPMOs), a longstanding barrier to applying Morpholino antisense techniques in adult animals has been overcome. Vivo-Morpholinos and PPMOs enter cells from the extracellular space and gain access to the cytosol and nuclear compartments. Antisense effects can be observed after systemic delivery of Vivo-Morpholinos or PPMOs.

1.1. Uses of unmodified Morpholinos

Effective techniques for delivering the oligos to the cytosol and nuclear compartments in tissue cultures have been developed, such as mechanical scraping [2], electroporation [3] or use of endosomal escape reagents [4]. Unmodified Morpholinos have been used routinely to block translation, modify splicing, inhibit miRNA activity and inhibit viral replication as well as more exotic RNA-blocking applications [5]. The Morpholino antisense structural type has been a revolutionary tool in developmental biology [6]. Success in embryos came by microinjecting Morpholino oligos into eggs or single or few celled zygotes, so that during cell division the Morpholinos were apportioned into daughter cells. This neatly avoided the problem of delivering the antisense separately into each cell of a many-celled organism [7]. In addition to their use to determine gene functions and interactions in embryos, Morpholino antisense oligos have been used for research into a broad range of diseases as well as several clinical trials (AVI BioPharma, Inc.). In most cases, carrying work from research to therapeutic applications of unmodified Morpholino oligos has been limited by difficulties with *in vivo* delivery [8]. However, delivery of relatively high doses of unmodified Morpholinos into dystrophic muscle in animal models of DMD has induced expression of functional Dystrophin in skeletal muscle [9,10] and unmodified Morpholinos are currently in clinical trial for treatment of DMD (http://clinicaltrials.gov/ct2/show/NCT00844597).

1.2. Morpholino chemistry and nomenclature

Morpholino oligos are manufactured from ribosides. The ribose ring is opened by oxidation, re-closed on ammonia and the product subsequently reduced to substituted morpholine. The base and the morpholine nitrogen are protected and the subunit is activated with a dimethylamino phosphoro-dichloridate. The activated subunits are added to a synthesis resin with washing, deprotection and activation steps for each activated base added. Oligos are cleaved from the resin and deprotected with ammonium hydroxide, then purified and quantitated, often followed by lyophilization and sterilization [11]. Substitutions such as peptides or the dendrimer scaffold for guanidinium may be added to the 3' morpholine nitrogen while the oligo is still attached to the synthesis resin. Alternatively, peptides may be added in the solution phase after resin cleavage and purification steps.

The ends of a Morpholino oligo are described as 3' and 5', but these labels do not refer to properly numbered atoms of the Morpholino backbone. Instead the atom designations of natural nucleic acids are used by analogy to label Morpholino ends; nucleic acids have a 5'-methylene hydroxyl (often phosphorylated) at one end and a 3'-ring hydroxyl at the other end. The methylene attached to the morpholine ring is designated as the 5' end of a Morpholino subunit and the morpholine nitrogen is designated as the 3' end; this nomenclature is chosen in order to make designation of direction along

the Morpholino oligo familiar to molecular biologists, who are accustomed to referring to the 5' and 3' ends of RNA and DNA.

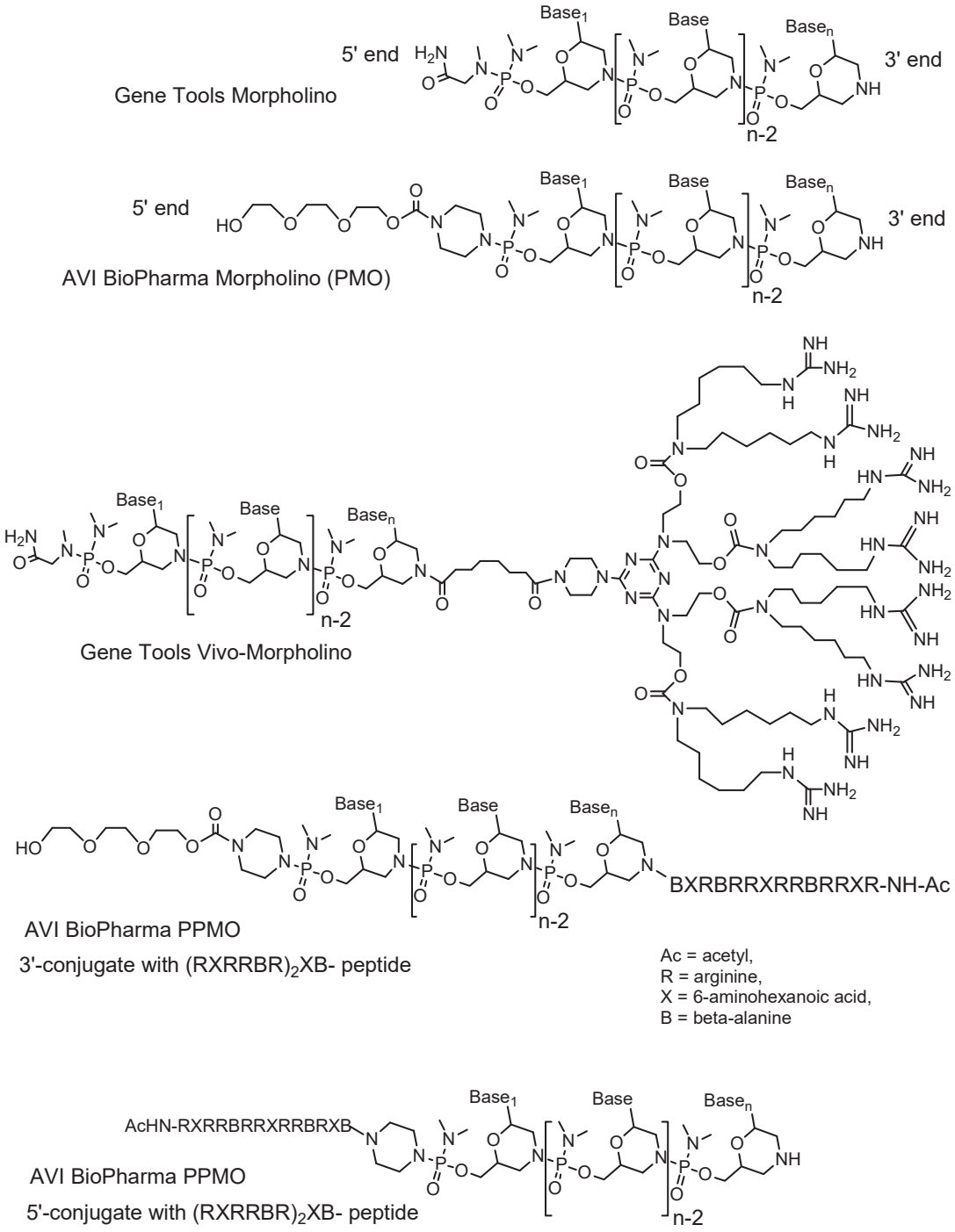
1.3. Endocytosis and the barrier to entry into the cytosol/nuclear compartment

Unmodified Morpholino oligos are endocytosed but have little to no activity against their RNA targets. Uptake by endocytosis does not mean the antisense oligos are reaching the cytoplasm or nucleus of the cell. Fluorescently-labeled Morpholinos can be detected as dim punctuate fluorescence within endosomes, but in most cells too little antisense escapes from the endosomes to the cytosol/nuclear compartment to be biologically active [2]. Morpholinos are not degraded in endosomes [12] but remain trapped. Many biological problems are best studied in adults and so a method that allows entry of antisense into most or all cells in an adult organism is very desirable. Ideally this systemic delivery could be accomplished by routine means such as intravenous or intraperitoneal injections. A technology that allows enough of the endocytosed oligos to escape from endosomes to have biological activity would enable *in vivo* delivery of oligos administered by systemic injection. Such technologies have now been developed: PPMOs and Vivo-Morpholinos. A PPMO may have an arginine-rich cell-penetrating peptide linked to either the 3' end or the 5' end of the Morpholino oligo [13]. The arginines have guanidinium moieties as part of their side chains, and the presence of these guanidiniums have been shown to increase cellular uptake of conjugated materials [14]. Studies of the mechanism of PPMO entry have shown that the cell-penetrating peptides offer two advantages over unmodified Morpholinos: enhanced uptake into endosomes and, critically, enhanced ensodomal escape [15]. A Vivo-Morpholino has an octaguanidinium dendrimer constructed on the 3' end of a Morpholino oligo [16] (Figure 1).

1.4. PPMOs

The first effective chemically-mediated method for systemic delivery of Morpholino antisense was based on covalently linking the oligos to cell-penetrating peptides, using arginine-rich cell-penetrating peptides to deliver uncharged antisense molecules [17]. These molecules are called peptide-linked phosphorodiamidate Morpholino oligomers, or PPMO for short. Research to develop more safe and effective peptide sequences for PPMOs has been the focus of Hong Moulton's group at AVI BioPharma Inc. [18,19], where preclinical work on PPMOs for Duchenne muscular dystrophy is currently ongoing. Studies on PPMOs have shown that uptake of the oligos is an energy-dependant and temperature-dependant process that can be prevented using endocytosis inhibitors; these characteristics indicate that uptake of the PPMOs is by endocytosis [20]. A fraction of endocytosed PPMOs escape from the endosome, entering the cytosol and nuclear compartment where they can block mRNA translation and modify pre-mRNA splicing [21]. Intravenous injection [22], intraperitoneal injection or intranasal administration [23] of PPMOs to mice have inhibited viral replication. Injections of PPMOs into transgenic mice carrying engineered splice-reporter genes have triggered expression of green fluorescent protein throughout the tissues [24].

Figure 1. Structures of unmodified and delivery-enabled Morpholino oligos.



Positively-charged arginine amino acid residues are not electrostatically attracted to uncharged antisense such as Morpholino oligos or peptide nucleic acid (PNA) oligos, so the positively charged arginines are free to interact with membranes [25]. At the end of an arginine amino acid's side chain is a guanidinium group, which carries the amino acid's positive charge. This charged guanidinium group is not only attracted to a phosphate because of the phosphate's negative charge, it can also form two hydrogen bonds with the phosphate's oxygens. This makes the attraction of a guanidinium and a

phosphate an unusually strong non-covalent interaction [26]. It may be this strong interaction that distorts the endosomal membrane and renders it permeable.

1.5. Vivo-Morpholinos

Vivo-Morpholinos are Morpholino antisense oligos covalently linked to a molecular scaffold that carries a guanidinium group at each of its eight tips. These custom-sequence antisense molecules enable Morpholino applications in adult animals. Vivo-Morpholinos have been shown effective in mice [27,28] and are being tested in rats, adult zebrafish and various organ explants.

To make a Vivo-Morpholino, the scaffold that will hold the guanidinium groups is added to the morpholine nitrogen at the 3'-end of a Morpholino oligo while the oligo is still bound at its 5' end to the synthesis resin. Subsequent treatment with ammonia cleaves the Vivo-Morpholino from the synthesis resin, de-protects the Morpholino's bases and de-protects the eight tips of the 3'-terminal scaffold. Next, treatment with *O*-methyl isourea converts the eight terminal amino groups to guanidinium groups [16].

2. Targets and outcomes of Morpholino experiments

Morpholino oligos bind to complementary RNA. If a molecular process normally occurs at the site where the Morpholino is bound to RNA, the Morpholino might be suitably positioned to get in the way of that process and prevent it from occurring. This mechanism is called steric blocking [29]. The effect of a Morpholino on a cell depends on where the Morpholino binds to the RNA, which determines whether it can block a process and which process it blocks. There are three common applications for Morpholino oligos: blocking translation of mRNA, modifying splicing of pre-mRNA or inhibiting miRNA activity [5]. In addition, work has been done on viral targets such as internal ribosomal entry sites [3] and cyclization sequences [30]; much of the reported work with PPMOs has involved viral targets [31]. Exotic targets have also been explored, such as binding to slippery sites to trigger ribosomal frameshifting [32] or binding to ribozymes to prevent catalytic cleavage of RNA [33].

2.1. Translation blocking

Translation blocking is the simplest method for knocking down gene expression with a Morpholino. When making a protein by cap-dependant translation, the small subunit of a ribosome binds to the 5' cap of an mRNA and, along with some other proteins, forms an initiation complex. The initiation complex moves along the 5' untranslated region (UTR) of the mRNA until a start codon is reached. At the start codon, the large subunit of the ribosome docks to small ribosomal subunit and then the process begins of linking together amino acids to form the new protein. Morpholinos that block the journey of the ribosomal initiation complex from the 5'-cap to the start codon, halting translation of the mRNA before the linking of amino acids can start. These Morpholinos can bind to RNA targets anywhere in the 5'-UTR through the start of the coding region, so long as the oligo binds onto the start codon or upstream (to the 5' direction) of the start codon [34].

The activity of a translation blocking oligo is assayed by an immunochemical method, typically Western blotting. Halting translation of a protein will not immediately cause a detectable change in the protein's signal on a Western blot. Some preexisting protein must be degraded over time before the

protein signal decreases. The time needed between knockdown and assay varies with the protein, as different proteins have different stabilities in cells.

2.2. Splice modification

After a pre-mRNA is transcribed from a DNA template, it goes through processing in the nucleus to form a mature mRNA. This processing typically includes adding a 5' cap, splicing out introns and ligating together exons, and adding a 3' poly-A tail. Splice modification involves binding Morpholinos to targets on a pre-mRNA (an unspliced mRNA) to redirect the spliceosome to a new pattern of splicing and produce a mature mRNA sequence that differs from the sequence produced in the absence of the Morpholino. Usually Morpholinos are bound to mostly-intronic targets at the boundaries between exons and introns. These targets prevent the binding of molecules that direct the spliceosome to splice sites. Typical results of splice modification include removal of a targeted exon or inclusion of the first or last intron in the mature mRNA [35].

Another class of targets consists of the binding sites on RNA where splice-regulation proteins normally bind, such as exonic splice enhancers or intronic splice suppressors. These proteins are important in directing alternative splicing, a natural process by which cells make alternative forms of mRNA from a single gene. For some targets, preventing a splice-regulatory protein from binding is an efficient method by which Morpholino can change the pattern of RNA splicing and therefore change the sequence of a mature mRNA [10,36].

Splice modification might produce an mRNA that is expressed as an altered protein and that protein might retain some activity and might still bind antibodies that would also bind to the unaltered protein. Because of this, immunochemical techniques used to detect knockdowns by translation blocking Morpholinos often do not work when used for detecting activity of splice-modifying Morpholinos. The activity of splice-modifying oligos is typically assayed by RT-PCR from primers that bind on either side of the altered sequence. If the altered splice leaves downstream sequence in-frame, the RT-PCR product will appear at a different location on an electrophoretic gel. Activation of a cryptic splice site can result in an RT-PCR fragment of unexpected size [37]. If the altered splice shifts the downstream reading frame, appearance of a premature termination codon often results in nonsense-mediated decay of the modified RNA, so the wild-spliced RT-PCR product band might dim on the electrophoretic gel without the corresponding splice-modified RT-PCR product appearing as a new band in a different position.

2.3. Inhibiting microRNA

A mature micro-RNA (miRNA) is a short strand of RNA bound to a protein complex which changes the expression of other genes by mechanisms that include cleaving or inhibiting translation of the target RNAs. We will discuss miRNAs of animals; there are some differences in plants. The target RNA must have sites at least partially complementary to the miRNA. Usually (but not always [38]) the target sites for miRNAs are in the 3'-UTR of mRNAs. miRNA is formed when a short stem-loop (hairpin) with the appropriate geometry forms in a newly-transcribed RNA. In the nucleus, the hairpin is recognized and cleaved by the Drosha nuclease, which cleaves both strands of the stem and leaves a few single-stranded overhanging bases. The resulting stem-loop is exported to the cytosol where it is recognized and cleaved by the Dicer nuclease, which removes the loop and also leaves a few single-

stranded overhanging bases. The resulting short double-stranded RNA is loaded onto a protein complex called the RNA-induced silencing complex (RISC), where a protein in the Argonaute family binds to one strand, called the guide strand, and cleaves the other strand. The cleaved strand dissociates and diffuses away, leaving an active miRNA-RISC complex [39].

Morpholinos can bind to the miRNA guide strand on RISC, preventing the guide strand from recognizing its targets on mRNAs [40]. Morpholinos can bind to the immature RNA hairpin, preventing Drosha from releasing the stem-loop from the rest of the transcript. Morpholinos can bind to the stem-loop, preventing Dicer from cleaving the loop from the stem [41]. A typical strategy is to design a 25-base Morpholino to bind the entire guide strand sequence (around 21 bases) and slightly into the loop sequence, allowing both inhibition of mature miRNA activity on RISC and inhibition of miRNA maturation by blocking Dicer. Another option is protecting a mRNA target. By binding to its complementary miRNA, a Morpholino blocks RISC from accessing the mRNA, thus relieving translation of the mRNA from inhibition by the miRNA and protecting the mRNA from cleavage by miRNA-directed Argonaute activity [42]. An advantage of target protection is that only the expression of the target gene is altered, while inhibiting maturation and activity of a miRNA can alter the expression of many mRNAs.

3. PPMOs

PPMOs are covalent conjugates of Morpholino oligos with cell-penetrating peptides. The peptide may be attached at the 3' or 5' end of the Morpholino oligo. Successful cell-penetrating peptides have contained arginine residues. The peptides may be composed of the alpha amino acids common in natural proteins or may contain other amino acids such as β-alanine or 6-aminohexanoic acid. Some cell-penetrating peptide sequences discussed in this section include $(RXR)_4B$-, $(RXR)_4XB$-, $(RXRRBR)_2XB$- and $(RX)_8B$-, with the dash at the peptide carboxy terminus representing the link to the Morpholino oligo. For these structures, R = arginine (L-arginine unless otherwise noted), B = β-alanine, and X = 6-aminohexanoic acid. Chiral amino acids may be in D or L forms, with the D forms chosen to resist proteolytic degradation, as in the two terminal arginines of the peptide $(DR)_2R_2QR_2K_2RF_2C$-. A procedure for solution-phase conjugation of Morpholino antisense oligos to arginine-rich peptides has been published [21].

The literature of PPMO therapeutic applications has been reviewed recently [8]. This section will focus on PPMO papers published since that review was prepared, especially seeking efficacy comparisons between unmodified Morpholinos and PPMOs and assessments of toxicity or immunogenicity of the PPMOs. Where many sequences of cell-penetrating peptides were reported in a paper, we will discuss the most effective of the peptides; for more detailed structure-activity comparisons, refer to the primary papers.

3.1. Screening cell-penetrating peptides in EGFP mice

A set of 14 cell-penetrating peptides were attached to the 5' ends of Morpholinos targeting a mutant splice site in human β-globin (IVS2-654) and administered to EGFP-654 mice, which carry a transgene with EGFP interrupted by an aberrantly-spliced human β-globin mutant intron (IVS2-654). Administration of different peptides resulted in different biodistributions. Because the cell-penetrating peptide $(RXRRBR)_2XB$- was effective at correcting IVS2-654 splicing in heart, diaphragm and

quadriceps, important target muscles for Duchenne muscular dystrophy (DMD) treatment, and did not cause toxicity at 12 mg/kg daily for four days, it was selected for intensive study. This PPMO also modified splicing in smooth muscles of the gut [24].

To determine persistence of effects, 12 mg/kg daily for four days of the $(RXRRBR)_2XB$- PPMO targeting IVS2-654 was administered to a group of EGFP-654 mice and mice were sacrificed and assessed periodically for 12 weeks. As shown by RT-PCR of cardiac muscle one day after the final injection, 90% of transcripts were splice-corrected. This decreased to 10% splice correction at six weeks. In diaphragm, splice correction decreased from 100% at 2-3 weeks after treatment to 10%-40% at 12 weeks. In quadriceps, splicing was still 100% corrected at 12 weeks. [24]

3.2. Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD) arises from some mutations of the human dystrophin gene. A potential treatment for this disease is the use of steric-blocking oligos to redirect splicing, skipping exons to remove early stop codons or to correct the reading frame disrupted by frameshift mutations. Several mouse models of DMD have been developed, the first and most popular being the *mdx* mouse, which has a premature stop codon in exon 23. Unmodified Morpholino antisense is currently in clinical trial for DMD. However, unmodified Morpholinos do not enter the heart at effective concentration and affect dystrophin splicing, even after repeated doses as high as 100 mg/kg in *mdx* mice [9]. For the three papers reviewed below, measurements of mRNA exon 23 skipping and Dystrophin concentrations after systemic delivery of PPMOs in *mdx* mice are summarized in Table 1.

3.2.1. Studies with the $(RXRRBR)_2XB$- cell-penetrating peptide PPMOs

A PPMO with the $(RXRRBR)_2XB$- peptide conjugated to the 3' end of a Morpholino sequence designed to cause splice-mediated excision of dystrophin exon 23 was injected iv into *mdx* mice at 12 mg/kg daily for four days. RT-PCR of RNA from heart tissue found that 70% of the dystrophin mRNA lacked exon 23 one day after the last treatment, decreasing to 50% at two weeks and 20% at seven weeks. At nine weeks after treatment, exon 23-skipped mRNA was still detected. In diaphragm and quadriceps, almost complete skipping of exon 23 persisted through nine weeks [24].

In-gel immunostaining revealed that in heart 30% of normal dystrophin levels were reached at 2-3 weeks and 15% remained at seven weeks after treatment. In diaphragm and quadriceps 40%-50% of normal dystrophin remained up to 17 weeks after treatment [24].

A PPMO with the $(RXRRBR)_2XB$- peptide on a Morpholino targeting dystrophin exon 23 in the *mdx* mouse was compared with an unmodified Morpholino of the same sequence. Either the Morpholino or the PPMO were injected im at 30 mg/kg into the tibialis anterior (TA) of adult *mdx* mice. The PPMO induced strong dystrophin expression in 85% of the fibers of the TA while the Morpholino induced dystrophin expression in 14% of TA fibers [43].

Table 1. Comparison of systemic delivery of PPMOs for DMD.

PPMO with $(RXRRBR)_2XB$- peptide on 3' end [24], 12 mg/kg iv tail vein, daily for 4 d	
Dystrophin **mRNA** with exon 23 skipped	Dystrophin **protein** concentration
Heart: 70%, 1d post-treat; 50%, 2wk post-treat; 20%, 7 wk post-treat. Diaphragm & quadriceps: ~100% thru 9 wk post-treat.	Heart: 30% dystrophin 2-3wk post-treat; 15% dystrophin 7wk post-treat. Diaphragm & quadriceps: 40%-50% dystrophin, 17 weeks post-treat.
PPMO with $(RXRRBR)_2XB$- peptide on 5' end [43], 30 mg/kg iv retro-orbital, single dose	
Dystrophin **mRNA** with exon 23 skipped	Dystrophin **protein** concentration
Heart: 63%, 2wk post-treat. Skeletal muscle: 80%-86%, 2wk post-treat.	Heart: 58% dystrophin, 2wk post-treat. Skeletal muscle: 91%-100% dystrophin, 2wk post-treat.
PPMO with $(RXRRBR)_2XB$- peptide on 5' end [43], 30 mg/kg iv retro-orbital, once every two weeks for three months (six times)	
Dystrophin **mRNA** with exon 23 skipped	Dystrophin **protein** concentration
Heart: 72%, 2wk post-treat. Skeletal muscle: 85%-92%, 2wk post-treat.	Heart: ~100% 2wk post-treat. Skeletal muscle: ~100% 2wk post-treat.
PPMO with $(RXR)_4XB$- peptide [44], 25 mg/kg iv tail vein, single dose	
Dystrophin **mRNA** with exon 23 skipped	Dystrophin **protein** concentration
Heart: 50%, 3wk post-treat. Skeletal muscle: Near 100%, 3wk post-treat.	Heart: 10%-20% dystrophin, 3wk post-treat. Skeletal muscle: 25%-100% dystrophin, 3wk post-treat.

Percent protein and mRNA are compared with wild-type muscle as 100%.

Single retro-orbital iv injections of the 30 mg/kg PPMO were administered to *mdx* mice and dystrophin expression was assessed two weeks later. Similar injections of the unmodified Morpholino induced dystrophin expression in 5% of skeletal muscle fibers. The PPMO induced strong dystrophin expression in 100% of skeletal muscle fibers and near-normal dystrophin levels were found by Western blot. RT-PCR of PPMO-treated mice showed 80%-86% of dystrophin transcripts in skeletal muscle were missing exon 23, demonstrating PPMO activity. Unmodified Morpholino induced no detectable dystrophin expression in cardiac muscle fibers. The PPMO induced dystrophin expression in 94% of cardiac muscle fibers and 58% of normal dystrophin levels was found by Western blot. RT-PCR revealed that 63% of dystrophin transcripts in cardiac muscle were missing exon 23 [43].

To assess longer-term treatment, *mdx* mice were iv injected retro-orbitally with PPMO at 30 mg/kg once every two weeks for three months, totaling six injections per mouse. Two weeks after the last injection, dystrophin was found in 100% of muscle fibers including smooth muscle of the small intestine. Dystrophin levels resembled those of normal mouse tissue. In cardiac muscle exon skipping

was improved by this dosing regimen, with 72% of dystrophin mRNA lacking exon 23 and strong dystrophin protein expression comparable to normal heart [43].

No damage to kidney or liver was detected by histological exams after PPMO treatment. Alkaline phosphatase and creatinine were not altered by PPMO treatment. Inflammatory cells did not accumulate in muscles and no antibodies reactive with PPMO were found in serum, indicating that the PPMO was not immunogenic [43].

3.2.2. Study with the $(RXR)_4XB$- cell-penetrating peptide PPMO

Single tail vein injections of 25 mg/kg PPMO with peptide sequence $(RXR)_4XB$- and a Morpholino sequence designed to cause excision of exon 23 were administered to *mdx* mice. Three weeks later, skeletal muscles were immunostained and showed near normal levels of dystrophin in most muscles analyzed. Western blot analysis found dystrophin concentrations ranging between 25% to 100% of normal in skeletal muscles. RT-PCR revealed almost total skipping of exon 23 in all skeletal muscles analyzed [44].

In heart muscle RT-PCR showed that about 50% of dystrophin transcripts lost exon 23. Immunostaining revealed dystrophin-positive fibers were widely distributed. By Western blotting, dystrophin concentrations between 10% and 20% of normal levels in heart were found [44].

PPMOs with the cell-penetrating peptides $(RXR)_4XB$- and $(RXRRBR)_2XB$- were injected iv and their efficacy compared. While both delivered antisense activity to skeletal and cardiac muscle, the $(RXR)_4XB$- did so more effectively [44].

To detect toxicity, analysis of the histology of liver and kidney and assays of some serum components were performed on samples from *mdx* mice that had been treated with 25 mg/kg PPMO. No overt signs of tissue damage in kidney or liver were revealed in haematoxylin and eosin stained tissues and there was no change in the number of infiltrating cells compared with untreated *mdx* mice. In the PPMO treated mice, levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) went from levels typical of *mdx* mice to levels typical of normal control mice. Serum levels of urea and creatinine did not change with PPMO treatment [44].

Weekly 6 mg/kg tail vein injections of the same PPMO for three weeks resulted in less exon skipping than the single 25 mg/kg tail vein injections, with the clearest decreases in efficacy in diaphragm, abdominal wall muscles and heart [44].

3.3. β-thalassemia

β-thalassemia is a common inherited genetic disease of humans. The ability of PPMOs to redirect splicing in a specific splice mutant of human β-globin was assessed in heterozygous knock-in mice, in which two adult murine β-globin genes (β-major and β-minor) were deactivated. This study used the cell-penetrating peptide $(DR)_2R_2QR_2K_2RF_2C$- covalently linked to a Morpholino targeting the aberrant 5' splice site created by the mutation IVS2-654 (C>T). Blocking this splice site restores normal splicing of β-globin transcripts bearing this IVS2-654 (C>T) mutation. Mice were treated with 25 mg/kg injections with daily injections for four days followed by three rest days, then this cycle was repeated. The experiment terminated on day 19 [45].

No significant liver or kidney toxicity were detected in PPMO-treated mice compared to saline-treated mice by standard biochemical tests (AST, ALT, BUN, CREA), nor was there significant weight

loss. Inflammatory response was assessed by RT-qPCR of IFN-γ and LI-12a cytokines; there was no significant change detected in their expression comparing saline-injected with PPMO-injected mice. Treatment of mice with a boost of PPMO a month after they were initially primed with 12 to 16 doses of the PPMO did not result in an IL-12 response as assessed by immunoassay for IL-12 on sera from days 3, 7 and 28 after boosting. Remaining sera from days 3, 7 and 28 after boosting was tested by ELISA for antibodies to the cell-penetrating peptide; no such antibody was detected. Lymphocytes from mice that had been treated 10 months earlier with either saline or PPMO were cultured ex vivo, challenged with PPMO and tested for induction of IFN-γ or antibody to the cell-penetrating peptide; neither response was detected [45].

The level of β-globin restoration theoretically achievable under these experimental conditions was fairly low. First, the mice were heterozygous for the human knock-in, so the human hemoglobin could only be restored to 50% normal concentration from the human gene. The duration of the experiment was 19 days but murine erythrocytes normally reside in blood for 60 days, so only a maximum of 1/3 of the erythrocytes could be replaced by the end of the experiment. Combining this with the 50% expression of human β-globin from the heterozygotes, the fraction of hemoglobin containing human β-globin theoretically achievable by the end of the experiment drops just under 17%. The level of chimeric mouse-human hemoglobin reached about 1-5% of the total hemoglobin in treated mice by day 19 [45].

3.4. Antiviral applications

Typically the highest antiviral efficacies are achieved with pre-infection administration of the PPMO followed by a series of post-infection doses. Recent studies explicitly comparing effects of unmodified Morpholino oligos and PPMOs show that the PPMOs are far more effective *in vivo*. Viral applications of PPMOs have recently been reviewed [31,46] so only a few recent papers applying PPMOs to inhibit viruses will be considered here.

3.4.1 Dengue study, mice and cultured cells, 2008

PPMOs in this study used the $(RXR)_4B$- cell-penetrating peptide moiety. Efficacy of Morpholinos and PPMOs targeting Dengue virus were tested in Vero cells and in AG129 mice. Oligos targeting the 5’ terminal nucleotides (5’SL) or the 3’ cyclization sequence (3’CS) were tested along with scramble-sequence control oligos. PPMO with Morpholino sequences ranging from 14 to 30 bases were prepared and tested in Vero cells against the virus DENV-2 D2/IC-30PA; PPMOs with Morpholino components of 22-24 bases length most effectively decreased viral titer. When the effect of oligo length on PPMO uptake was tested by flow cytometry of cells treated with PPMOs of 18, 24 or 30 base length bearing fluorescent labels, no effect of length on uptake was shown and PPMOs of all lengths tested entered over 95% of the cells. Toxicology and pharmacokinetic studies on PPMOs were included with this study of antiviral efficacy of Morpholinos and PPMOs against Dengue virus. Dose regimens were tested for toxicity in AG219 mice before being used in separate AG219 mice to assess Dengue inhibition. Pharmacokinetic measurements and additional toxicity tests were done using BALB/c mice [47].

AG219 mice were treated by ip injection with daily doses of 380 μg of scramble-sequence Morpholino oligo (about 19.1 mg/kg) or 240 μg of scramble-sequence PPMO (about 12.1 mg/kg) for

two days, followed by monitoring over 35 days. No ill effects were observed and the animals maintained their weights. The same dosing regimen was used with oligo sequences targeting Dengue virus but between the two oligo doses the mice were infected with 10^6 pfu DENV-2 NGC. Treatment with 240 μg of a 5'SL-targeted 20-mer PPMO extended average survival by eight days and treatment with 240 μg of a 3'CS-targeted 18-mer PPMO extended average survival by five days when compared with control mice which were similarly infected but treated with PBS and averaged nine days survival. There was no survival benefit when mice were treated with 380 μg of unmodified Morpholino oligo targeting either 5'SL or 3'CS, nor after Morpholino or PPMO scramble-sequence treatment [47].

To determine toxicity of a longer dosing regimen, AG219 mice were treated with 10 mg/kg daily ip injection of 5'SL 22-mer for nine days and compared with a group receiving similar PBS injections. The animals increased in mass by an average of 1 g over the 35-day study regardless of treatment arm, with all animals maintaining very similar weights. This experiment was repeated but with 10^4 pfu Dengue infection between the second and third oligo doses. Treatment with a combination of 5'SL 22-mer and 3'CS 22mer extended life eight days on average and treatment with 5'SL 22-mer extended life seven days on average, while other treatments did not extend life by statistically significant periods compared to the 14 day survival of PBS-treated infected controls [47].

BALB/c mice were treated with fluorescein-conjugated PPMO 5'SL 24-mer in an intraperitoneal dose of 10 mg/kg. Pharmacokinetics and biodistribution into brain, liver and spleen were determined from measurements at 0.2, 0.5, 1, 2, 3, 8, 12, and 24 hours. Peak plasma concentration was 0.48 mg/L. A distribution half-life of 2.79 h and an elimination half-life of 7.31 h were determined. The liver accumulated the most PPMO-Fl the most rapidly while the brain accumulated the least. Toxicity in Balb/c mice was addressed for a nine dose regimen with daily ip injection of 10 mg/kg fluoresceinated 5'SL 24-mer PPMO. Animals appeared and behaved normally through the nine-day dosing and five-day washout period. Significant increase in serum cholesterol and decreases in haematocrit, haemoglobin, packed cell volume and red blood cell count were observed during treatment but returned to levels similar to control mice during the washout period. Total bilirubin did not increase during treatment, so treatment likely inhibited red blood cell production instead of increasing red blood cell lysis. The concentrations of fluoresceinated PPMO in liver and spleen did not change significantly over the washout period, demonstrating good retention in tissues after uptake [47].

3.4.2. Ebola study, mice and cultured cells, 2009

This study used oligos complementary to the ebola Zaire virus VP24 transcript, comparing unmodified Morpholinos and PPMOs with a different class of modified Morpholinos bearing a cationic moiety on some of their phosphorodiamidate linkages (PMO+). PPMOs in this study used several cell-penetrating peptide moieties, including $(RXR)_4XB$-, $(RX)_8B$- and $(RB)_8B$-. The PPMOs were shown to have higher efficacies than PMO+ or unmodified Morpholinos against ebola virus when tested in a cell free translation system, Vero E6 cell cultures and C57BL/6 mice. Some unmodified Morpholino sequences blocked translation in cell-free translation systems but these generally had no or limited efficacy in cell cultures or in mice [48].

Vero E6 cells were pre-treated with oligo for two hours, then infected with a GFP-expressing Zaire ebola virus. After 48 hours the cells were fixed and observed for fluorescence. Uninfected cells had very little autofluorescence. Infected untreated cells and infected cells pre-treated with bare

Morpholinos were clearly fluorescent. Infected cells pre-treated with PPMO had reduced fluorescence, and PPMO with peptide conjugated at the oligo's 5' end reduced fluorescence more effectively than PPMO with the peptide attached to the 3' end of the Morpholino moiety. Three different cell-penetrating peptides were tried, linked at the 5' end and the 3' end of the Morpholino; at 10 μM all of the PPMOs with 5'-linked cell-penetrating peptides inhibited viral replication more than 35% when compared to infected cells treated with unmodified Morpholino [48].

Mice were treated twice with PPMOs having $(RX)_8B$- peptide conjugated to Morpholinos targeting the 5'-terminal end of the VP24 transcript. Mice were injected at 24 hours prior and at four hours prior to infection with ebola virus. Lethal challenges of 1000 pfu ebola virus were delivered ip and the mice were tracked for 15 days. The PPMOs having $(RX)_8B$- peptide conjugated to the 5' end of the Morpholino were an effective treatment as doses of 50 μg or 5 μg kept all treated mice alive and doses of 1 μg saved 60% of mice tested. In a similar experiment with pretreatment at 24 hours prior and four hours prior to infection with 1000 pfu ebola virus ip, PPMOs constructed with fewer RX repeats were not as effective at protecting mice from ebola challenges and unmodified Morpholinos did not prevent death of the mice when used in this experiment at 50 μg per injection. Similar experiments using PPMOs having $(RX)_8B$- peptide conjugated to Morpholinos targeting the start codon of the VP24 transcript gave 100% protection at 50 μg per dose and 60% protection at 5 μg per dose when the oligos were conjugated at their 5' end and gave 95% protection at 50 μg per dose and 95% protection at 5 μg per dose when the oligos were conjugated at their 3' end. In this experiment, unmodified Morpholinos gave 15% protection at 50 μg per dose [48].

3.4.3. Influenza study, mice, 2008

Morpholinos conjugated with the $(RXR)_4XB$- cell-penetrating peptide were administered intranasally to Balb/C mice and inhibited viral replication. One oligo targeted the start site of viral polymerase subunit PB1 mRNA (PB1-AUG), while another targeted the 3' end of the NP virion RNA (NP-V3'); these were prepared as unmodified Morpholinos and as PPMOs. Intranasal delivery to the lung tissue was shown effective using a scrambled-sequence PPMO conjugated with fluorescein, by which delivery into the lung was shown to be best in the upper lung and closest to the major bronchioles and decreasing through the middle and into the lower lung. Toxicity testing was performed for treatment of up to 7.5 mg/kg administered intranasally once and again 24 hours later. Compared to saline-treated mice, PPMO-treated mice showed no significant weight loss, assessed by daily weighing over the 72 hours following the first dosing, and no observable illness. No significant differences were noted from histopathological examination of lung tissue compared to saline-treated mice. Leukocyte populations in spleen and lungs were followed by flow cytometric analysis; no significant changes in spleen populations were found and lung populations only differed significantly at the highest dose per administration (7.5 mg/kg, with no significant differences at doses of 3.75 mg/kg or less). At 7.5 mg/kg dosing, macrophages increased 2.21 fold and granulocytes increased 3.38 fold. The authors suggest that the arginine-rich cell-penetrating peptides may be causing leukocyte infiltration by mimicking a known effect of poly-arginine, which triggers a response similar to major basic protein, a component of the granules of eosinophils [49].

To test antiviral activity, mice were dosed intranasally with a 50:50 mixture of the two PPMOs, PB1-AUG and MP-V3', at 3.74 mg/kg in each dose administered at four hours before and 20 hours

after infection with influenza virus A/Eq/Miami/1/63 (H3N8). These mice were compared with mice dosed similarly with unmodified Morpholinos, with Morpholino and PPMO scramble sequence controls, or with saline. Lung tissue was taken at 72 hours after infection for plaque assays and the mixed PB1-AUG and MP-V3' PPMO was shown to reduce viral growth 1.5 $\log_{10}$ compared to scramble PPMO. The mixed PB1-AUG and MP-V3' unmodified Morpholinos caused slight but not significant decrease in viral titer compared to the scramble Morpholino. Tests comparing pre-infection treatment versus post-infection treatment showed clear benefit to pre-treatment, though smaller but still significant decreases in viral titer occurred with treatment delayed to 1 hr or 2 hr post-infection. [49]

3.5. Restenosis

Global Therapeutics, the cardiology unit of Cook Medical (http://www.cookmedical.com), is developing PPMO-coated stents for balloon angioplasty using PPMOs from AVI BioPharma Inc. The Morpholino moiety targets c-myc to inhibit proliferation of the vascular endothelial cells, which might otherwise cause restenosis.

4. Applications of Vivo-Morpholinos in animal studies

Vivo-Morpholinos are Morpholino oligos coupled with eight guanidinium head groups on dendrimer scaffolds that enable delivery into cells [16]. We and others have demonstrated that like PPMOs, Vivo-Morpholinos extend proven Morpholino antisense technology to research in adult animals. Potent antisense effects were observed in a wide range of tissues in animals injected with Vivo-Morpholinos at dosages that did not produce detectable toxicity [27,28]. Vivo-Morpholinos have been used successfully in various adult animals including healthy mice [27], a mouse model of Duchenne muscular dystrophy [27,28] and a dog model of Duchenne muscular dystrophy (Toshifumi Yokota, personal communication). Vivo-Morpholinos provide powerful tools for gene regulation studies in animals with an efficient synthesis designed for high-throughput production protocols [16].

4.1. Dramatically improved delivery of Vivo-Morpholinos versus unmodified Morpholinos in adult animals

Morpholino oligomers have been widely used to modulate gene expression in cell cultures and in embryos of zebrafish, frogs, tunicates, chicks and sea urchins [50]. Activity of unmodified Morpholino oligos in adult animals has been reported primarily in leaky tissues such as the damaged muscles in animal models of DMD [9,10,51]. However, the delivery efficiency is highly variable within tissues and inefficient in some tissues including the heart and diaphragm muscles [9]. Vivo-Morpholinos improved delivery from blood into the cytosol and nuclei of dystrophic mice more than 50 fold compared with unmodified Morpholinos, which only entered muscle at all due to the leakiness characteristic of DMD muscle [28]. In addition, the antisense effects of Vivo-Morpholinos were achieved evenly and efficiently in body-wide tissues including heart, diaphragm and smooth muscle [28]. In healthy adult animals, iv treatment with unmodified Morpholino oligomers does not cause antisense activity in cells [27]. Recent studies have shown that Vivo-Morpholinos achieve delivery in a wide variety of tissues in normal adult mice while no detectable delivery of unmodified Morpholino

was achieved at the same dosage [27]. In summary, Vivo-Morpholinos exhibit significantly higher delivery efficiency in adult animals than do unmodified Morpholinos.

4.2. Animal systems tested

The efficacy of antisense effect mediated by Vivo-Morpholinos has been tested in healthy adult mice and a DMD disease mouse model with leaky muscles. Vivo-Morpholinos were tested in adult mice with intact cell membranes bearing a transgene with a mutation preventing the production of enhanced green fluorescent protein (EGFP). This error could be corrected by redirecting splicing with a Vivo-Morpholino oligo, resulting in the expression of EGFP. Recent studies demonstrated that Vivo-Morpholinos efficiently corrected the error in various tissues of the EGFP transgenic mice and showed no detectable toxicity [27]. In the *mdx* mouse model of DMD, Vivo-Morpholinos have shown potent antisense effects in body-wide muscles including the hard-to-deliver heart and diaphragm muscles [27, 28]. Vivo-Morpholinos have been tested by local injection in a DMD disease dog model in which they exhibit similar potency as when locally injected into *mdx* mice (Toshifumi Yokota, personal communication).

4.3. Delivery method and efficacy in different tissues

Different routes of injection result in various biodistributions of Vivo-Morpholinos to tissues. Both local and systemic delivery methods have been tested in adult mice (Table 2) [27,28].

Table 2. Tissues that were tested for antisense effect in mice injected with Vivo-Morpholinos by different routes.

Injection Method	Effective tissues	Ineffective tissues	Reference
Subcutaneous (sc)	Skin	Skeletal muscles, heart	Jiang S, unpublished data
Intramuscular (im)	Muscles near injected site	Heart, diaphragm	[28]
Intraperitoneal (ip)	Skeletal muscles: diaphragm, abdominal, limb	Heart	[28]
Intravenous (iv)	Liver, small intestine, colon, skeletal muscles, spleen, lung, heart, skin, stomach, kidney.	Brain	[27, 28]

Subcutaneous (sc) injection is mainly effective for targeting skin and fails to deliver to the muscles and heart (Jiang S *et al*. manuscript in preparation). Intramuscular (im) injection efficiently delivers Vivo-Morpholinos to the muscles near the injected sites, but not in most other tissues including the heart, brain and diaphragm. Intraperitoneal (ip) injection is the most efficient route to deliver Vivo-Morpholinos to the diaphragm and the abdominal muscles [28]. By intravenous (iv) injection, systemic delivery was achieved in most tissues including liver, small intestine, colon, skeletal muscles, spleen, lung, heart, skin, stomach and kidney but not brain [27,28].

4.4. Vivo-Morpholinos in DMD studies and clinical relevance

The antisense efficacy of Vivo-Morpholinos was demonstrated in several Duchenne muscular dystrophy (DMD) disease animal models [27,28]. Recent data suggest that the efficacies of Vivo-Morpholinos and PPMOs are similar after local injections in dystrophic dogs (Toshifumi Yokota, personal communication). A Vivo-Morpholino targeting a splice site on the dystrophin mRNA restored Dystrophin protein production in *mdx* mice [27]. The efficiency of antisense effect was dosage dependent. Vivo-Morpholinos exhibit significantly higher impact on both splice-modified mRNA and protein levels at a dose of 25 mg/kg than at 12.5 mg/kg [27]. A single intravenous injection of 6 mg/kg Vivo-Morpholino spliced out the defect of the dystrophin gene in more than 50% of the skeletal muscle fibers of *mdx* mice [28]. Repeated injection of 6 mg/kg Vivo-Morpholinos into *mdx* mice biweekly over 10 weeks restored Dystrophin protein to near normal level in all skeletal muscles tested including biceps, diaphragm, intercostals, tibialis anterior, quadriceps, gastrocnemius, and abdominal muscles. Significantly, these injections of Vivo-Morpholinos achieved cardiac delivery and enabled Dystrophin protein production in the heart [28]. Assays of blood markers for liver toxicity (AST,ALT) showed no change of liver function [27] and no significant impact on the body weight or the histology of the liver and kidney [28]. Importantly for potential therapeutic applications, multiple injections of Vivo-Morpholinos did not stimulate an adaptive immune response in mice as determined by ELISA against Vivo-Morpholinos using mouse serum [28]. The potent antisense effect in animals and lack of obvious toxicity at the effective dosage make Vivo-Morpholinos good research reagents for modifying gene expression. The therapeutic index and long term toxicity must be assessed because periodic administration of Vivo-Morpholinos is often required to maintain the antisense effect.

5. Conclusions

Until recently, effective use of Morpholino antisense has been limited to applications where delivery techniques have been devised. There have been many attempted but few successful *in vivo* applications of unmodified Morpholinos in adult animals. Development of Morpholino oligos conjugated to cell-penetrating peptides (PPMO) has made adult animal experiments feasible for Morpholinos. Clinical development of PPMOs on stents to inhibit restenosis after balloon angioplasty is expected to begin imminently and PPMOs are in late preclinical development for Duchenne muscular dystrophy. Unlike PPMOs, Vivo-Morpholinos are commercially available as research reagents. Vivo-Morpholinos have been successfully tested in adult animals, providing investigators with convenient access to research-grade delivery-enabled Morpholino antisense.

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